Remarks

Claims 38-44, 46-66, 74-87, and 90-92 were pending in the subject application. By this Amendment, new claims 93 and 94 have been added. The undersigned avers that no new matter is introduced by this amendment. Entry and consideration of the amendments presented herein is respectfully requested. It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of the applicant's agreement with or acquiescence in the Examiner's position. Accordingly, claims 38-44, 46-66, 74-87, and 90-94 are currently before the Examiner for consideration. Favorable consideration of the pending claims is respectfully requested.

Submitted herewith is a Request for Continued Examination (RCE) under 37 C.F.R. §1.114 for the subject application. Also submitted herewith is a supplemental Information Disclosure Statement (IDS), accompanied by the form PTO/SB/08 and copies of the references listed therein. The applicants respectfully request that the references listed on the form PTO/SB/08 be considered and made of record in the subject application.

By this Amendment, claims 93 and 94 have been added. Support for these claims can be found, for example, at page 15, lines 29-34, and page 16, lines 25-30, of the specification.

Claims 38-44, 46-66, 74-87, and 90-92 have been rejected under 35 U.S.C. §112, first paragraph, as lacking sufficient written description and as constituting new matter. The applicant respectfully traverses and submits that the subject specification provides a sufficient written description of the claimed invention. Furthermore, the claimed subject matter does not represent new matter.

New Matter

At page 4, the Office Action indicates that the specification makes no specific mention of RNAi molecules or RNAi inhibition. This is not determinative of whether the claimed subject matter represents new matter. The applicant submits that the subject specification, taken as a whole, would lead one of ordinary skill in the art to use RNAi molecules that interfere with expression of SHIP-1 mRNA. Page 5, lines 1-24; page 7, lines 13-29; page 8, lines 3-4, 11-12, 17-19, and 21-25; page 9, lines 10-15; page 11, lines 8-9; page 12, lines 13-18; page 13, lines 23-24; and claims 1-5 of

the application as originally filed support this. The Examiner must consider the common usage of the term "interfering" at the time the subject application was filed, which was after the advent of RNAi. For the sake of clarity of the record, the applicant once again respectfully requests that the Examiner indicate whether the Examiner is taking official notice of the common usage of the term "interfering" in connection with transcription and/or translation of a target gene's RNA at the time the application was filed, within the meaning of 37 C.F.R. §1.104(d)(2); MPEP §2144.03. The applicant respectfully submits that the subject specification sets forth sufficient "blaze marks", as required in *In re Ruschig*, 379 F.2d 990; 154 USPQ 118 (CCPA 1967), to lead one of ordinary skill in the art to interfering RNA. Assuming *arguendo* that the term "means for interfering with transcription and/or translation of SHIP RNA" encompasses a genus of various inhibitors, interfering RNA would be singled out and immediately envisioned by one of ordinary skill in the art, based on the teachings of the subject specification as a whole.

The subject specification teaches that the inhibitor of SHIP-1 function can be a genetic construct, such as "an anti-sense oligonucleotide, an RNA aptamer capable of inhibiting SHIP enzymatic activity, an RNA aptamer capable of inhibiting a ribozyme, or another genetic construct of inhibiting SHIP activity known to those of skill in the art" (page 11, lines 10-15). The specification teaches that the substance that inhibits SHIP function can be a nucleic acid that hybridizes to a SHIP mRNA (page 5, lines 33-34; page 6, lines 26-27; and page 11, lines 25-26). The specification teaches that the delivered nucleic acid molecule can incorporate into a specified gene so as to inactivate the gene and "turn off" the product the gene was making, or to alter the translation or stability of the mRNA of the specified gene product (page 12, lines 13-16). The subject application teaches that the nucleic acid can be either RNA or DNA, may be a non-coding sequence, and may be single-stranded or double-stranded (page 14, lines 7-9 and 15). Furthermore, the specification teaches that the SHIP inhibitor can be DNA that directs production of RNA or a polypeptide that inhibits SHIP function (page 15, lines 33-34). There is no requirement that any one segment of the specification, standing alone, has to provide the full support for the claims. Rather, the assessment is to be made from the perspective of one of ordinary skill in the art at the time the application was filed, guided by the teachings of the specification as a whole. Based on the characteristics provided

in the subject specification, one of ordinary skill in the art would immediately envision interfering RNA as a means for inhibiting translation of SHIP-1 at the time the application was filed.

At page 4, the Office Action indicates that the post-filing submissions of previously published work concerning interfering RNA do not provide proper support for RNAi in the application as filed. These submissions were made to show what was known to those skilled in the art at the time the application was filed. This is germane to both the new matter rejection and the rejection for lack of written description, as such determinations are to be made from the perspective of those of ordinary skill in the art at the time the application was filed.

Finally, the applicant notes that claims 74-87 and claims 90-94 do not recite the term "interfering RNA" and find literal support in those portions of the specification set forth in the Amendments in which those claims were introduced.

Written Description

The Examiner indicates that the specification and claims do not adequately describe the concise structural features that distinguish structures within the claimed genus from those without (*e.g.*, the nucleotide sequences or a representative number of RNAi molecules of the generic RNAi structures claimed, which specifically bind and inhibit SHIP-1 function *in vivo*, and which suppress graft-versus-host disease and transplant rejection).

At page 6 of the Office Action, the Examiner notes that various "splice isoforms and sequence variants have been reported for mouse or human SHIP-1". The claims currently recite that the administered interfering RNA, polynucleotide, nucleic acid, or DNA reduces SHIP-1 function or expression in mouse or human hematopoietic cells, rendering some of the isoforms/variants irrelevant. There would be no difficulty in identifying target mRNA sequences shared by all known hematopoietic SHIP-1 isoforms in humans and mice, due to the extensive amount of sequence overlap between the isoforms (see Figure 2A of Rohrschneider *et al.*, *Genes & Development*, 2000, 14:505-520). Furthermore, as stated previously, hematopoietic SHIP-1 sequences have the enzymatic domain (inositol 5'-phosphatase). One of ordinary skill in the art would likely consider the SHIP-1 enyzmatic domain as the starting point for selecting target sequences and corresponding inhibitory nucleic acids.

The test for determining whether a claimed invention is adequately described in the specification is whether the originally filed disclosure reasonably conveys to a person of ordinary skill in the art that the applicant had possession of the subject matter claimed. As acknowledged in the Office Action, the specification provides an adequate written description of the target SHIP-1 mRNA recited in the claims. Having the sequence of the target gene (SHIP-1) and knowledge of its structure, including its relevant isoforms, at the time of filing, one skilled in the art could readily envision target nucleic acid sequences within and along the recipient mammal's mRNA. Furthermore, nucleic acid molecules likely to hybridize with SHIP-1 mRNA and interfere with its expression could then be determined. Due to the certainty of the genetic code and complementarity, there is a well known correlation between target nucleic acid sequences within a target gene and nucleic acid sequences that interfere with the expression of the target gene. The following publications were previously made of record: International Publication WO 99/32619 (Fire *et al.*), Tuschl T. *et al.* (*Genes & Development*, 1999, 13:3191-3197); Zamore P. *et al.* (*Cell*, 2000, 101:25-33); Svoboda P. *et al.* (*Development*, 2000, 127:4147-4156); Tuschl, T. *et al.* (*Chembiochem*, 2001, 2(4):239-245); Elbashir S. *et al.* I (*Nature*, 2001, 411:494-498); Elbashir S. *et al.* II (*Genes & Development*, 2001, 15:188-200) and Caplen N.J. *et al.* (*PNAS*, 2001, 98(17):9742-9747). RNAi is triggered by dsRNA and results in sequence-specific degradation of homologous single-stranded target RNAs. When dsRNA containing a sequence complementary to a specific mRNA target is administered to cells, it is processed into short nucleotide fragments that guide the cleavage of the transcript. Thus, the endogenous mediators of RNAi are short (*e.g.*, 21-23-nucleotide) interfering RNAs (siRNAs) generated from the longer double-stranded RNAs by the ribonuclease III activity of the highly conserved dicer enzyme (Tuschl T. *et al.* (1999); Zamore P. *et al.*; Elbashir S. *et al.* I; and Elbashir S. *et al.* II). It has been demonstrated that RNAi-mediated gene suppression can be obtained in mammalian cells by delivery of chemically synthesized short (*e.g.*, less than 30 nucleotides) double-stranded siRNA molecules or by endogenous expression of short hairpin RNAs (shRNAs) bearing a fold-back stem-loop structure (Elbashir *et al.* I).

The interfering RNA and hybridizing nucleic acid molecules recited in the claims are not described by function alone. As is evidenced by the aforementioned publications, structural attributes of interfering RNA, including size and content, were known in the art at the time the

application was filed (see, for example, pages 197-198 of Elbashir S. *et al.* II). Elbashir *et al.* proposed directly introducing short (*e.g.*, 21-23 nucleotides) dsRNA (siRNA) into mouse and human cells to avoid the problems associated with the expression of longer dsRNAs (Elbashir S. *et al.* I). Elbashir *et al.* state:

> The finding that synthetic 21- and 22-nt siRNA duplexes can be used for efficient mRNA degradation demonstrates that the targeting step can be uncoupled from the dsRNA-processing step. This raises the prospects of using siRNA duplexes as new tools for sequence-specific regulation of gene expression in functional genomics as well as biomedical studies. The siRNA may be effective in mammalian systems, where long dsRNAs cannot be used because they activate the dsRNA-dependent protein kinase (PKR) response (Clemens 1997). As such, the siRNA duplexes may represent a new alternative to antisense or ribozyme therapeutics. (Elbashir S. *et al.* II, page 198, column 2)

Hence, having the nucleotide sequence of the target gene provides discerning information regarding the sequences (*i.e.*, structural information) of suitable interfering nucleic acid molecules, and leads one of ordinary skill in the art to their selection. Accordingly, the teaching of the subject specification and knowledge of the sequence and structure of the SHIP-1 gene provides sufficient structural and functional correlates to describe the genus of target mRNA and corresponding interfering RNA and hybridizing nucleotides. The Office Action appears to acknowledge that the state of the art at the subject application's filing date was sufficiently developed such that the design of RNAi molecules for inhibiting expression of a target gene *in vitro* is a "routine technique", requiring only "routine experimentation" (see the Examiner's stated basis for the rejection under 35 U.S.C. §103(a), set forth at page 10, lines 16-22, of the previous Office Action dated September 26, 2006).

The written description requirement states that the applicant must describe the invention; it does not state that every invention must be described in the same way. The applicant acknowledges that sequences and structural formulas provide a convenient method of demonstrating possession of many molecules; however, other identifying characteristics or combinations of characteristics may demonstrate the requisite possession. An applicant may show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that the applicant was in possession of the claimed invention, *i.e.*, complete or partial structure, other

physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. MPEP§ 2163. In *Enzo Biochem, Inc. v. Gene-Probe, Inc.*, 63 USPQ2d 1609 (Fed Cir. 2002), the Court reaffirmed that deposit of a physical sample may replace words when description is beyond present scientific capability. In *Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 65 USPQ2d 1385 (Fed Cir. 2003), the Court explained further that the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." For example, possession of an antibody may be demonstrated based on a description and characterization of its corresponding antigen. Disclosure of an antigen fully characterized by its structure, formula, chemical name, physical properties, or deposit in a public depository provides an adequate written description of an antibody claimed by its binding affinity to that antigen. *Noelle v. Lederman*, 69 USPQ2d 1508, 1514 (Fed. Cir. 2004) and MPEP 2163 IIA3(a).

At page 7, the Office Action indicates that the ability to screen candidate inhibitory molecules for their ability to inhibit a target gene *in vitro* is "merely an invitation to experiment further to identify RNAi that exhibit inhibitory activity". An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). There is no *per se* rule that an actual reduction to practice must occur prior to filing, or that the need to screen for candidate nucleic acid molecules precludes adequate written description of the nucleic acid molecules. Possession may be shown in a variety of ways, including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. See, *e.g.*, MPEP §2163.02, *Pfaff v. Wells Elecs., Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997); *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it"). Compliance with the written

description requirement is a fact-based inquiry that will necessarily vary depending on the nature of the invention claimed. *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 323 F.3d 956, 963; 63 USPQ2d 1609, 1613 (Fed. Cir. 2002).

Due to their nature, the interfering RNA and other nucleic acid molecules recited in the claims are clearly distinguishable from the compounds at issue in *University of Rochester v. G.D. Searle & Co.*, 69 USPQ2d 1886 (Fed. Cir. 2004), for example, in which the Court affirmed that the description of the cyclooxygenase-2 enzyme (COX-2) and an assay for identifying selective inhibitors of COX-2 did not provide an adequate written description of unknown non-steroidal molecules capable of selectively inhibiting the enzyme. The teaching of the subject specification and the knowledge of the sequence and structure of the SHIP-1 gene provide one skilled in the art with a sufficient structural template and functional correlates to describe the genus of interfering RNA and hybridizing nucleic acid molecules that suppress expression of the SHIP-1 gene in human or mouse hematopoietic cells. The subject specification does not require the screening of vast amounts of candidate small molecules *de novo*, based on function alone, with no guidance provided or available as to the molecular structure of a receptor agonist to be identified. Rather, the teaching of the subject specification, the knowledge of the sequence and structure of the SHIP-1 gene, and the mechanism by which the recited molecules inhibit gene expression, together provide sufficient structural and functional correlates to demonstrate possession of the interfering RNA and hybridizing nucleotides recited in the claims. Identification of specific interfering RNA and specific hybridizing nucleotides would not just be likely, it would be inevitable and imminent. All functional descriptions of genetic material do not necessarily fail to meet the written description requirement as a matter of law. Rather, the Court has held that the written description requirement may be satisfied if, in the knowledge of the art, the disclosed function is sufficiently correlated to a particular, known structure. *Enzo Biochem, Inc.* Such is the case here. The written description requirement must be considered in the context of the claimed invention and the state of knowledge in the relevant art. *Capon et al. v. Eshhar et al.*, 418 F.3d, 1349 (Fed. Cir. 2005).

The fundamental concept of the invention is that SHIP-1 deficiency would be of therapeutic benefit in suppressing transplant rejection and graft-versus-host disease (GVHD), as taught in the subject application. Furthermore, the applicant has shown that only partial SHIP-1 deficiency in the

myeloid lineage is required to achieve significant suppression of allogeneic T cell responses, which mediate GVHD and graft rejection. The state of the art was sufficiently developed such that tools for inducing the required SHIP-1 deficiency were appreciated by the inventor, taught in the patent application, and available to those of ordinary skill in the art. Thus, the applicant submits that the patent application contains sufficient disclosure to convey to one of ordinary skill in the art that the applicant had possession of the concept of what is claimed, which is all that is necessary to satisfy the written description requirement of 35 U.S.C. §112, first paragraph. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. §112, first paragraph, is respectfully requested.

Claims 38-44, 46-66, 74-87, and 90-92 have been rejected under 35 U.S.C. §112, first paragraph, as non-enabled by the subject specification. The applicant respectfully submits that the claims are fully enabled by the subject specification.

The previous Office Action acknowledged that the state of the art at the subject application's filing date was sufficiently developed such that the design of RNAi molecules for inhibiting expression of a target gene *in vitro* is a "routine technique", requiring only "routine experimentation" (see page 10, lines 16-22, of the previous Office Action). Furthermore, the Office Action indicates that the specification provides enablement for a method of suppressing the rejection of an allogeneic bone marrow graft from BALB/C mice in $SHIP^{-/-}$ mice or abrogating GVHD in $SHIP^{-/-}$ mice that were transplanted with whole bone marrow from BALB/C mice, thereby enhancing $SHIP^{-/-}$ mouse survival, and for the *in vivo* inhibition of SHIP-1 expression in mice using the RNAi sequences #1 and #4, and the mouse antisense vector muSHIPshRNA provided in the Declarations by Dr. Kerr, filed July 21, 2004, and February 9, 2005. However, the Office Action indicates that the patent application does not provide enablement for inhibiting SHIP-1 *in vivo*, comprising administering any RNAi molecule specific for SHIP-1 mRNA present in mouse or human hematopoietic cells, or administering *in vivo* or *ex vivo* any nucleic acid molecule that hybridizes *in vitro* under conditions of stringency with human or mouse SHIP-1 mRNA or that hybridizes *in vivo* with SHIP-1 mRNA present in mouse or human hematopoietic cells, or suppressing transplant rejection in any patient, or treating GVHD in any patient, comprising administering any interfering RNA molecule specific for SHIP mouse or human mRNA. Specifically, the Office Action indicates that one skilled in the art would not accept the ability of co-administered RNAi molecules, or the mouse antisense vector

muSHIPshRNA to target and successfully inhibit expression of the SHIP-1 gene in a mouse model, and provide an observed increase in Mac+Gr1 monocytes and circulating Mac1+GR1+ cells (myeloid suppressor cells), as representative or correlative of the claimed subject matter,

> in view of the lack of guidance in the specification and known unpredictability associated with the ability to predict the efficacy of interfering RNA in inhibiting the expression of SHIP ... following administration by any route of the claimed RNA oligonucleotides" (paragraph bridging pages 8-9 of the Office Action).

As the Examiner is aware, a specification is initially presumed to be in compliance with the enablement requirement of §112, first paragraph. The burden is on the Patent Office to establish a reasonable basis to question enablement. *In re Wright*, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). The test of enablement is whether one of ordinary skill in the art could make and use the claimed invention from the teachings of the patent application, coupled with information known in the art, without undue experimentation. For an Office Action to sustain a rejection on the grounds of enablement, it must provide evidence or a scientific basis for the assertion that the claimed invention could not be made or used without undue experimentation. The applicant notes that the Office Action does not state what "guidance" is missing from both the subject specification and the knowledge of those skilled in the art at the time the application was filed that is allegedly necessary to carry out the invention without resort to undue experimentation. However, it appears that the delivery of nucleic acids to cells *in vivo* with commensurate treatment effects is the main issue.

The applicant has submitted several scientific publications demonstrating successful delivery and activity of antisense oligonucleotides *in vivo* (Tong *et al.*, *Clin. Lung Cancer*, 2001, 2(3):220-226, abstract only; Lau *et al.*, *Antisense Nucleic Acid Drug Dev.*, 2002, Feb., 12(1):11-20; Prasad *et al.*, *Anticancer Res.*, 2002, Jan.-Feb., 22(1A):107-116, abstract only; Eder *et al.*, *Cancer Gene Ther.*, 2002, Feb., 9(2):117-125; Miyake *et al.*, *Clin. Cancer Res.*, 2001, 7(12):4245-4252; Choi *et al.*, *J. Clin. Invest.*, 2001, 108(12):1833-1841; Marchand *et al.*, *Am. J. Physiol. Heart Cir. Physiol.*, 2002, Jan., 282(1):H194-204; Ueta *et al.*, *Int. J. Cancer*, 2001, 94(4):545-550; Wang *et al.*, *Clin. Cancer Res.*, 2001, 7(11):3613-3624; Olson *et al.*, *Clin. Cancer Res.*, 2001, 7(11):3598-3605; Uchida *et al.*, *Mol. Urol.*, 2001, 5(2):71-78, abstract only; Tortora *et al.*, *Clin. Cancer Res.*, 2001, 7(8):2537-2544;

Berg *et al.*, *J. Pharmacol. Exp. Ther.*, 2001, 298(2):477-484; Frankel *et al.*, *Cancer Res.*, 2001, 61(12):4837-4841; and Finotto *et al.*, 2001, *J. Exp. Med.*, 193(11):1247-1260).

Furthermore, the applicant submits that while the currently pending claims of the subject application are fully enabled, it is art-recognized that RNAi differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference requires delivery to a cell interior of specific-single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. RNAi has advantages over antisense both in the stability of the material to be delivered and the concentration required for effective inhibition (see page 2, lines 12-29, and page 4, lines 14-25, of International Publication WO 99/32619 (Fire *et al.*). Furthermore, compared to antisense or ribozyme technology, the secondary structure of the target mRNA does not appear to have a strong effect on RNAi-mediated silencing (see Harborth J. *et al.*, *J. Cell Sci.*, 2001, Dec., 114 (Pt. 24):4557-4565). In fact, RNAi has now become such a popular tool for gene silencing that many companies now use proprietary algorithms to design and chemically synthesize siRNAs using a conventional DNA/RNA synthesizer. Research groups have created human shRNA libraries that target thousands of genes and used them to identify new genes (see pages 80-81 and 84 of Bonetta, L. "RNAi: Silencing never sounded better" *Nature Methods*, 2004, 1(1):79-86). Moreover, since the subject application was filed, RNAi-mediated gene silencing *in vivo* has been demonstrated in non-human primates (Zimmermann T.S. *et al.*, *Nature*, 2006, 441(7089):111-114; Tolentino M.J. *et al.*, *Retina*, 2004, 24:132-138, which are submitted herewith).

The use of gene delivery vehicles, such as vectors, for administration of SHIP-1 inhibitory substances, were contemplated at the time of filing and disclosed at page 11, lines 19-25, and page 12 of the specification.

> The delivery vehicle can be any component or vehicle capable of accomplishing the delivery of a gene or a substance to a cell, for example, a liposome, a particle, naked DNA, or a vector. A gene delivery vehicle is a recombinant vehicle, such as a recombinant viral vector, a nucleic acid vector (such as plasmid), a naked nucleic acid molecule such as a gene, a nucleic acid molecule complexed to a polycationic molecule capable of neutralizing the negative charge on the nucleic acid molecule and condensing the nucleic acid molecule into a compact molecule, a nucleic acid associated with a liposome (Wang, *et al.*, *PNAS* 84:7851, 1987), and certain

> eukaryotic cells such as a producer cell, that are capable of delivering a nucleic acid molecule having one or more desirable properties to host cells in an organism. The desirable properties include the ability to express a desired substance, such as a protein, enzyme, or antibody, and/or the ability to provide a biological activity, which is where the nucleic acid molecule carried by the gene delivery vehicle is itself the active agent without requiring the expression of a desired substance (page 11, line 34; and page 12, lines 1-13, emphasis added).
>
> One example of such biological activity is gene therapy where the delivered nucleic acid molecule incorporates into a specified gene so as to inactivate the gene and "turn off" the product the gene was making, or to alter the translation or stability of the mRNA of the specified gene product. Gene delivery vehicle refers to an assembly which is capable of directing expression of the sequence(s) or gene(s) of interest or of turning off the gene of interest (page 12, lines 13-18).

Hairpin expression vectors have been used for delivery of RNAi to mammalian cells, as demonstrated by Yu *et al.* (*PNAS*, 2002, Apr., 99(9):6047-6052), Paddison *et al.* (*PNAS*, 2002, Feb., 99(3):1443-1448); Abbas-Terki *et al.*, *Hum. Gene Ther.*, 2002, Dec., 13(18):2197-2201); Svoboda *et al.*, *Biochem. Biophys Res. Commun.*, 2001, 287(5):1099-1104); and Ma *et al.* (*Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, 2002, Sept., 16(3):253-355, abstract only), which have been previously submitted. Cationic liposomes such as DOTAP are positively charged and interact with the negatively charged DNA molecules to form a stable positively charged DNA/liposome complex, which is internalized by the cell. Cationic lipids such as DOTAP have been used as nucleic acid delivery vehicles for some time (see, for example, column 17 of U.S. Patent No. 6,025,198, Bennett *et al.*, "Antisense Modulation of SHIP-2 Expression", of record; Porteous *et al.*, "Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis", *Gene Ther.*, 1997, 4(3):210-218, previously submitted; Song *et al.*, "Characterization of cationic liposome-mediated gene transfer *in vivo* by intravenous administration", *Hum. Gene Ther.*, 1997, 8(13):1585-1594, abstract only, previously submitted; and Ott *et al.*, "A cationic sub-micron emulsion (MF59/DOTAP) is an effective delivery system for DNA vaccines," *J. Controlled Release*, 2002, 79:1-5, previously submitted). Since the application was filed, systemic administration of interfering RNA has proven effective (Soutschek J. *et al.*, *Nature*, 2004, 432(7014):173-178, which is submitted herewith).

Consideration is to be given to post-filing date evidence (*e.g.*, Declarations and Exhibits) offered by the applicant to show that the claimed invention works, provided that the evidence is consonant with the teachings of the specification as filed. In making this determination, the Examiner is to compare the materials and methods used in the experiments of the Declaration and Exhibits with those taught in the application to make sure that they are commensurate in scope. This means that the Examiner is to confirm that the experiments used the guidance in the specification as filed and what was well known to one of skill in the art (MPEP §2164.05). Thus, the requirement of consonance between the submitted evidence and the teachings of the specification is not evaluated in a vacuum. Rather, the determination is to be made from the standpoint of one of ordinary skill in the art. Thus, the knowledge possessed by those persons of ordinary skill in the pertinent art of nucleic acid delivery, for example, must be considered. A disclosure in an application, to be complete, must contain such description and details as to enable any person skilled in the art or science to which the invention pertains to make and use the invention as of its filing date. *In re Glass,* 492 F.2d 1228, 181 USPQ 31 (CCPA 1974). As iterated in MPEP 608.01(p), the prior art setting may be mentioned in general terms. It is "the essential novelty, the essence of the invention, [that] must be described in such details, including proportions and techniques, where necessary, as to enable those persons skilled in the art to make and utilize the invention."

The enablement requirement of 35 U.S.C. §112, first paragraph, does not require that the applicant reinvent the wheel. There is no need to inform the layman nor disclose what one of ordinary skill in the art already possesses.

> Paragraph 1 permits resort to material outside of the specification in order to satisfy the enablement portion of the statute because it makes no sense to encumber the specification of a patent with all the knowledge of the past concerning how to make and use the claimed invention. One skilled in the art knows how to make and use a bolt, a wheel, a gear, a transistor, or a known chemical starting material. The specification would be of enormous and unnecessary length if one had to literally reinvent and describe the wheel. *Amtel Corporation v. Information Storage Devices, Inc.*, 198 F.3d 1374; 53 USPQ2d 1225 (Fed. Cir. 1999).

As the Examiner is aware, the quantity of experimentation needed to be performed by one skilled in the art is only one factor involved in determining whether "undue experimentation" is required to make and use the invention. "[A]n extended period of experimentation may not be undue

if the skilled artisan is given sufficient direction or guidance." *In re Colianni*, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977). "'The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.'" *In re wands*, 858 F.2D 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (citing *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)). Time and expense are merely factors in this consideration and are not the controlling factors. *United States v. Telectronics Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989). MPEP §2164.06. "Tedious and laborious" experimentation is not necessarily undue experimentation for purposes of enablement under 35 U.S.C. §112, first paragraph. *Ex parte Erlich* 3 USPQ2d 1011 (BPAI 1982).

The SHIP-1 knockout models utilized in the Examples of the subject application and Dr. Kerr's Declarations demonstrate that SHIP-1 deficiency results in a phenotype that is of therapeutic benefit, and that this phenotype is achieved in the absence of complete SHIP-1 silencing. The evidence of record shows that nucleic acid molecules, such as interfering RNA, can be successfully delivered to cells *in vitro* and *in vivo*, and achieve the required level of SHIP-1 knockdown established with the SHIP-1 knockout models.

The applicant previously submitted the unpublished Paraiso *et al.* manuscript entitled "Induction of SHIP deficiency prior to allogeneic bone marrow transplant enhances engraftment and survival", of which Dr. Kerr is a co-author. The manuscript demonstrates that induction of SHIP-deficiency in the adult allogeneic bone marrow transplant recipient enhances both the quality and duration of their post-transplant survival. When taken with the other experimental evidence submitted with the applicant's previous responses, it is clear that: (1) SHIP deficiency can be induced just prior to engraftment and still result in enhanced transplant survival; (2) even partial SHIP deficiency will enhance transplant survival; and (3) nucleic acid molecules, such as interfering RNA can be administered to a human or mouse recipient using known delivery methods to achieve the required SHIP deficiency, without resort to undue experimentation.

The applicant respectfully submits that, in view of the state of the art of nucleic acid delivery at the time the application was filed, one of ordinary skill in the art would be able to make and

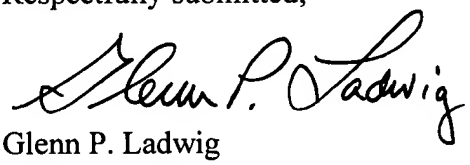
deliver agents, such as interfering RNA, to human cells *in vitro* and *in vivo*, without the need for undue experimentation. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

In view of the foregoing remarks and amendments to the claims, the applicant believes that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

The applicant invites the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs

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RNA interference (RNAi) holds considerable promise as a therapeutic approach to silence disease-causing genes, particularly those that encode so-called 'non-druggable' targets that are not amenable to conventional therapeutics such as small molecules, proteins, or monoclonal antibodies. The main obstacle to achieving *in vivo* gene silencing by RNAi technologies is delivery. Here we show that chemically modified short interfering RNAs (siRNAs) can silence an endogenous gene encoding apolipoprotein B (apoB) after intravenous injection in mice. Administration of chemically modified siRNAs resulted in silencing of the apoB messenger RNA in liver and jejunum, decreased plasma levels of apoB protein, and reduced total cholesterol. We also show that these siRNAs can silence human apoB in a transgenic mouse model. In our *in vivo* study, the mechanism of action for the siRNAs was proven to occur through RNAi-mediated mRNA degradation, and we determined that cleavage of the apoB mRNA occurred specifically at the predicted site. These findings demonstrate the therapeutic potential of siRNAs for the treatment of disease.

RNAi has been applied widely as a target validation tool in post-genomic research, and it represents a potential strategy for *in vivo* target validation and therapeutic product development[1]. *In vivo* gene silencing with RNAi has been reported using both viral vector delivery[2] and high-pressure, high-volume intravenous (i.v.) injection of synthetic siRNAs[3], but these approaches have limited if any clinical use. *In vivo* gene silencing has also been reported after local, direct administration (intravitreal, intranasal and intrathecal) of siRNAs to sequestered anatomical sites in models of choroidal neovascularization[4], lung ischaemia-reperfusion injury[5] and neuropathic pain[6], respectively. These reported approaches demonstrate the potential for delivery to organs such as the eye, lungs and central nervous system. However, there are no published reports of systemic activity for siRNAs towards endogenous targets after conventional and clinically acceptable routes of administration. A critical requirement for achieving systemic RNAi *in vivo* is the introduction of 'drug-like' properties, such as stability, cellular delivery and tissue bioavailability, into synthetic siRNAs.

Conferring drug-like properties on siRNAs

In exploring the potential of synthetic siRNAs to silence endogenous target genes, we found that chemically stabilized and cholesterol-conjugated siRNAs[7] have markedly improved pharmacological properties *in vitro* and *in vivo*. Chemically stabilized siRNAs with partial phosphorothioate backbone and 2'-*O*-methyl sugar modifications on the sense and antisense strands showed significantly enhanced resistance towards degradation by exo- and endonucleases in serum and in tissue homogenates. The conjugation of cholesterol to the 3′ end of the sense strand of a siRNA molecule by means of a pyrrolidine linker (thereby generating chol-siRNA) did not result in a significant loss of gene-silencing activity in cell culture. Furthermore, unlike unconjugated siRNAs, a chol-siRNA directed to luciferase (chol-luc-siRNA) showed reduction in luciferase activity in HeLa cells transiently expressing luciferase, with a half-maximal inhibitory concentration (IC_{50}) of about 200 nM in the absence of transfection reagents or electroporation.

Binding of chol-siRNAs to human serum albumin (HSA) was determined by surface plasmon resonance measurement (data not shown). Unconjugated siRNAs demonstrated no measurable binding to HSA, whereas chol-siRNAs bound to HSA with an estimated dissociation constant (K_d) of 1 μM. Presumably because of enhanced binding to serum proteins, chol-siRNAs administered to rats by i.v. injection showed improved *in vivo* pharmacokinetic properties as compared to unconjugated siRNAs. After i.v. injection in rats at 50 mg kg^{-1}, radioactively labelled chol-siRNAs had an elimination half life (two compartments), $t_{1/2}$ of 95 min and a corresponding plasma clearance (C_L) of 0.5 ml min^{-1}, whereas unconjugated siRNAs had a $t_{1/2}$ of 6 min and C_L of 17.6 ml min^{-1}. As measured by an RNase protection assay (RPA), chol-siRNAs showed broad tissue biodistribution 24 h after injection in mice. Although no detectable amounts of unconjugated siRNAs were observed in tissue samples, significant levels of chol-siRNAs were detected in liver, heart, kidney, adipose, and lung tissue samples. Together, these studies demonstrate that cholesterol conjugation significantly improves *in vivo* pharmacological properties of siRNAs.

Selection of apoB as an endogenous gene target

Apolipoprotein B is the essential protein for formation of low-density lipoproteins (LDL) in metabolism of dietary and endogenous cholesterol, and is the ligand for the LDL receptor[8]. Mouse apoB is a large protein of 4,515 amino acids and is expressed predominantly in liver and jejunum. apoB mRNA is subject to post-transcriptional editing, and the unedited and edited transcripts encode the full-length protein apoB-100, and a carboxy-terminal truncated isoform, apoB-48, respectively. In mice, editing of apoB mRNA occurs in both the liver and jejunum: apoB-48 is the predominant protein form in the jejunum and both apoB-48 and apoB-100 are expressed in the liver. Heterozygous knockout mice for apoB show a 20% decrease in cholesterol levels and are resistant to

diet-induced hypercholesterolaemia[9].

Serum levels of apoB, LDL and cholesterol correlate significantly with increased risk of coronary artery disease (CAD). A diminished number of functional LDL receptors on the cell surface, disrupting receptor-mediated removal of apoB-containing LDL from circulation, has been identified as the basis for familial hypercholesterolaemia (FH)[10]. Patients with homozygous and heterozygous FH have accelerated CAD leading to premature atherosclerosis and cardiac mortality. Conversely, patients with hypobetalipoproteinaemia have reduced levels of LDL and cholesterol and are at reduced risk for CAD[11]. Accordingly, lowering of serum cholesterol and LDL levels is a predominant clinical strategy for management of CAD and is achieved by modification of dietary sources of cholesterol and/or inhibition of endogenous cholesterol synthesis with pharmacological therapies. Notwithstanding significant improvements in the management of CAD with these approaches, millions of patients remain at significant risk for CAD and its clinical sequelae—acute coronary syndromes such as myocardial infarction and cardiac mortality—due to advanced atherosclerosis from intractably high levels of cholesterol and LDL. Clearly, new therapeutic strategies are needed. Accordingly, apoB, a protein not amenable to inhibition by conventional small-molecule- or protein-based therapeutics, was selected as a potential clinical target for development of siRNA therapeutics.

Using conventional bioinformatics, 84 siRNAs specific for both human and mouse apoB mRNA were designed and synthesized (data not shown). These apoB-siRNAs were screened for their ability to reduce apoB mRNA and protein levels, as measured by polymerase chain reaction with reverse transcription (RT–PCR) and enzyme-linked immunosorbent assay (ELISA), respectively, in HepG2 liver cells after transfection at a concentration of 100 nM. Five apoB-siRNAs were identified that reduced both mRNA and protein levels by >70%. Because exonucleolytic degradation is the predominant mechanism for siRNA degradation in serum, two selected apoB-siRNAs (apoB-1-siRNA and apoB-2-siRNA) and one four-nucleotide mismatch control for apoB-1-siRNA (mismatch-siRNA) were stabilized at the 3′ end of the sense and antisense strands by phosphorothioate backbone modifications and additional incorporation of two 2′-*O*-methyl nucleotides at the 3′ end of the antisense strand. Chol-siRNAs were synthesized by linkage of cholesterol to the 3′ end of the sense strand via a pyrrolidine linker. Chol-apoB-1-siRNA was significantly more stable than unconjugated apoB-1-siRNA in human serum: gel electrophoresis showed >50% intact chol-apoB-1-siRNA after a 1 h incubation at 37 °C compared with <5% intact unconjugated apoB-1-siRNA. Similar data were obtained for chol-apoB-2-siRNA, although this siRNA was less stable than chol-apoB-1-siRNA. Dose response curves for the activity of conjugated and unconjugated apoB-specific and control siRNAs were measured in HepG2 cells using transfection. Two conjugated control siRNAs (chol-luc-siRNA and chol-mismatch-siRNA) showed no significant inhibition of apoB protein expression at concentrations as high as 30 nM. In contrast, three specific siRNAs (unconjugated apoB-1-siRNA, chol-apoB-1-siRNA and chol-apoB-2-siRNA) showed dose-dependent silencing of apoB protein expression based on apoB ELISA measurements—IC_{50} values of 0.5 nM, 5 nM and 8 nM were calculated, respectively.

In vivo studies with modified siRNAs

To demonstrate the ability of chol-apoB-siRNAs to silence apoB expression *in vivo*, experiments were first performed in C57BL/6

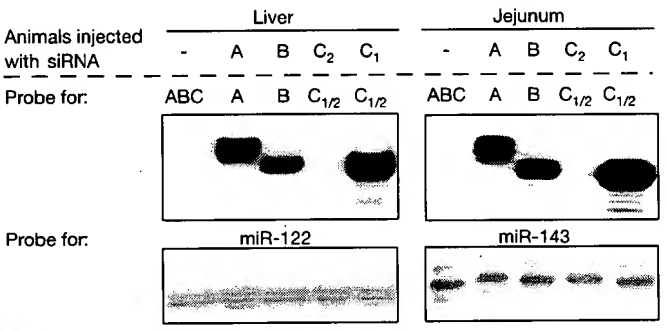

Figure 1 Biodistribution of siRNAs in liver and jejunum. An RPA was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals injected with saline (−), chol-luc-siRNA (A), chol-mismatch-siRNA (B), unconjugated apoB-1-siRNA (C_2) or chol-apoB-1-siRNA (C_1). Detection by RPA of endogenous miRNAs in liver (miR-122) and jejunum (miR-143) served as an internal loading control.

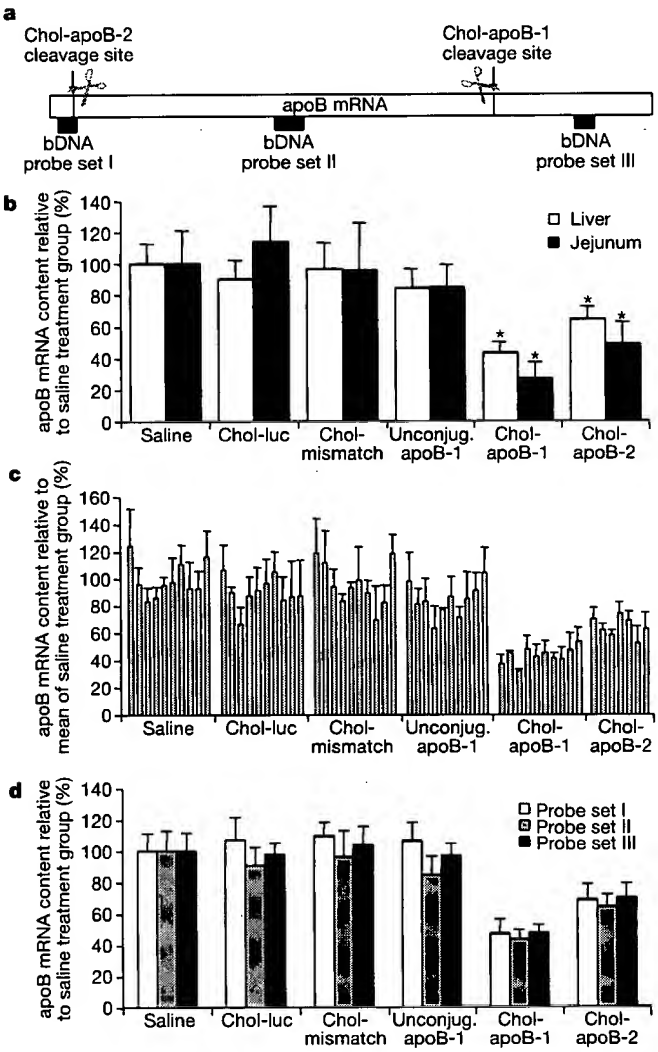

Figure 2 *In vivo* silencing of murine apoB mRNA by siRNAs in wild-type mice. Treatment groups comprised saline control ($n = 10$), chol-luc-siRNA control ($n = 10$), chol-mismatch-siRNA control ($n = 10$), unconjugated apoB-1-siRNA ($n = 10$), chol-apoB-1-siRNA ($n = 10$) and chol-apoB-2-siRNA ($n = 7$). bDNA measurements were performed with probe set II. Error bars represent the standard deviation (s.d.) of the mean. Statistical analysis was by analysis of variance (ANOVA) with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline control animals. **a**, Schematic representation of the apoB mRNA illustrating the binding regions of three bDNA probe sets in relation to the two siRNA cleavage sites. **b**, Effects of siRNA administration on mean apoB mRNA levels. **c**, apoB mRNA levels from individual mice treated with saline or siRNAs. Data are mean values from three liver samples from each individual animal. **d**, Effects of siRNA administration on the reduction of apoB mRNA measured by bDNA assays using three different probe sets.

mice fed a normal chow diet. siRNAs were administered by tail-vein injection with normal volume (0.2 ml) and normal pressure. Biodistribution of siRNAs was assessed by RPA of siRNAs in tissue samples from liver and jejunum obtained 24 h after the last injection. Significant levels of chol-luc-siRNA, chol-apoB-1-siRNA and chol-mismatch-siRNA were detected in liver and jejunum (100–200 ng g^{-1} tissue for chol-apoB-1-siRNA), whereas levels of unconjugated apoB-1-siRNA were below our detection limit (Fig. 1). Levels of chol-apoB-2-siRNA were also detected but at levels approximately 10% of those observed for other chol-siRNAs.

The primary measure of RNAi-mediated effects is the reduction (that is, silencing) of the target mRNA. To measure silencing of apoB mRNA, we used a branched-DNA (bDNA) detection method and bDNA probes (Fig. 2a) to quantify apoB mRNA levels in liver and jejunum, two organs where apoB is known to be expressed. As shown in Fig. 2b, mice treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed statistically significant reductions (mean ± s.d.; 57 ± 6% and 36 ± 8%, respectively) in apoB mRNA levels in liver samples as compared with saline control ($P < 0.0001$). In jejunum tissue samples, mice injected with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed an even more substantial reduction in apoB mRNA levels of 73 ± 10% and 51 ± 13%, respectively, as compared with saline control ($P < 0.0001$). Individual animal results for apoB mRNA reduction in the liver are shown in Fig. 2c and demonstrate the consistent and robust effect observed for specific chol-siRNAs as compared with other treatment groups. Similar results were observed for apoB mRNA reduction in the jejunum from individual animals (data not shown). Owing to the extended length of the apoB mRNA, two additional probes at the distal ends of the apoB open reading frame (ORF) were designed. As measured with the three divergent probe sets, identical levels of apoB mRNA reduction were detected for animals treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA (Fig. 2d). These data suggest a uniform and rapid degradation of apoB mRNA after treatment with chol-apoB-siRNAs, and argue against the potential existence of truncated amino-terminal apoB protein fragments translated from incompletely degraded siRNA-cleavage products, as has been reported for ribozyme-mediated cleavage of apoB mRNA[12].

Silencing of the apoB mRNA would be expected to result in a corresponding reduction in apoB protein levels. An ELISA-based method specific for detection of apoB-100 protein was used to measure the effects of chol-apoB-siRNA treatment on plasma levels of apoB protein. In addition to the effects on apoB mRNA levels, treatment with chol-apoB-1-siRNA and chol-apoB-2-siRNA reduced plasma levels of apoB-100 protein 24 h after siRNA treatment by 68 ± 14% and 31 ± 18%, respectively, compared with levels in saline-treated control animals (Fig. 3). These results achieved statistical significance ($P < 0.0001$) for the group treated with the more potent and stable chol-apoB-1-siRNA. As the LF3 antibody used in this study recognizes only apoB-100, and not apoB-48, the observed apoB-100 reduction may underestimate the full effect of chol-apoB-1-siRNA at the protein level.

To confirm the physiological relevance of apoB mRNA silencing on lipoprotein metabolism, we characterized the effect of siRNA treatment and the resulting reduction of apoB protein levels on lipoprotein profiles and cholesterol levels. Using an NMR-based method, complete lipoprotein profiles were generated and concentrations of chylomicrons, very-low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL) particles were calculated (Fig. 4a). As expected, HDL represented the predominant lipoprotein fraction in mouse plasma. Similar to results observed in heterozygous knockout mice for apoB[9], treatment with chol-apoB-1-siRNA resulted in a 25% reduction in HDL particle

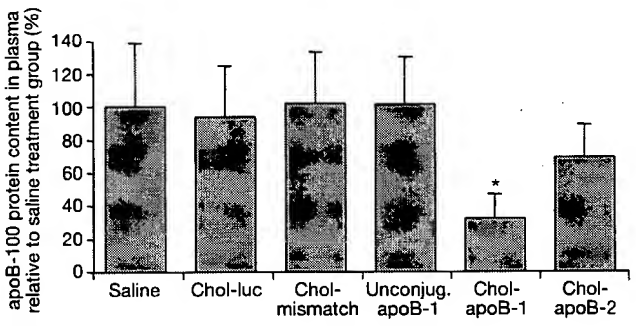

Figure 3 Effects of siRNA administration on apoB-100 protein levels. Average plasma levels of apoB-100 protein for the different treatment groups as measured by ELISA. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline control animals.

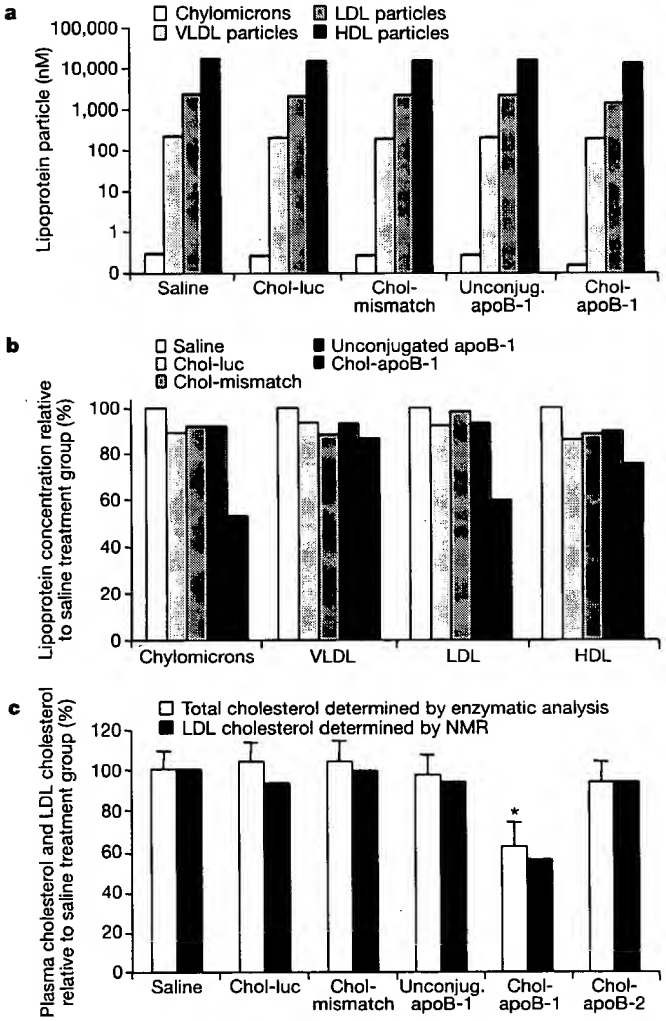

Figure 4 Therapeutic reduction of lipoprotein and cholesterol levels after siRNA treatment. **a**, Lipoprotein profile of pooled plasma samples from treatment groups determined by NMR analysis. **b**, Relative reduction of lipoprotein classes for the siRNA treatment groups normalized against the average levels of saline control group. **c**, Effects of siRNA administration on plasma cholesterol and LDL cholesterol. Plasma cholesterol was determined by enzymatic assay and LDL cholesterol calculated from NMR measurements. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline and chol-mismatch-siRNA control animals. NMR data are based on single measurements of pooled plasma from treatment groups.

concentration (Fig. 4b). Furthermore, treatment of mice with chol-apoB-1 siRNA resulted in an almost 50% reduction of chylomicron levels and an approximately 40% reduction in LDL levels, whereas VLDL levels were not altered. Treatment with either of the control siRNAs did not change the lipoprotein profile significantly. In addition to reductions in lipoprotein concentrations, *in vivo* silencing of apoB by chol-apoB-1-siRNA led to a significant reduction ($37 \pm 11\%$; $P < 0.0001$) of total plasma cholesterol as compared with saline control animals (Fig. 4c). Treatment with the less potent chol-apoB-2-siRNA failed to show significant reductions in cholesterol, consistent with the reduced activity of this chol-siRNA on apoB mRNA and protein levels. Treatment with chol-apoB-1-siRNA also resulted in a 44% decrease in LDL-associated cholesterol, consistent with the effects observed on apoB protein levels. In aggregate, the effects on cholesterol reduction and lipoprotein profiles would be considered highly clinically significant in patients with hypercholesterolaemia, and actually exceed the level of cholesterol reduction observed in heterozygous apoB knockout mice[9].

To extend our findings of *in vivo* silencing by chol-apoB-siRNAs in normal mice, we performed an additional study in a human apoB transgenic mouse model[13]. These mice express human apoB-100 in liver and have elevated levels of apoB as compared with normal mice; when fed a high-fat diet, these mice develop severe atherosclerosis[14]. In our experiments, we administered saline, chol-mismatch-siRNA and chol-apoB-1-siRNA to apoB transgenic mice fed a normal chow diet. As shown in Fig. 5, chol-apoB-1-siRNA brought about a significant reduction of endogenous murine apoB expressed in both liver and jejunum tissue samples ($P < 0.0001$, relative to saline and chol-mismatch-siRNA treatment). Relative to the saline control, levels of murine apoB mRNA were reduced by $57 \pm 10\%$ in liver and $42 \pm 12\%$ in jejunum. In addition, chol-apoB-1-siRNA, which was selected in part owing to its sequence identity to both human and mouse apoB, showed significant silencing of the human transgene expressed in the liver, where human apoB mRNA was silenced by $60 \pm 10\%$ ($P < 0.0001$). In contrast to these effects, chol-mismatch-siRNA showed no effect on mouse or human apoB mRNA levels. These results confirm the effect of specific chol-siRNAs on apoB silencing in a different mouse model. Moreover, this specific chol-siRNA was shown to silence a transgenic human mRNA *in vivo*.

An important consideration for siRNA-mediated inhibition of gene expression is whether the observed effects are specific and not due to nonspecific "off target" effects[15] and potential interferon responses[16], which have been reported with siRNAs *in vitro* and other oligonucleotide-based approaches *in vivo*. In our experiments, the effects of apoB-specific, cholesterol-conjugated siRNAs were seen with two divergent siRNAs targeting separate sequence regions of the apoB mRNA. Furthermore, the *in vivo* silencing of apoB by these siRNAs was specific as neither an irrelevant siRNA (chol-luc-siRNA) nor a mismatch control siRNA (chol-mismatch-siRNA)—although present at comparable concentrations in liver and jejunum—mediated a significant reduction in apoB mRNA, plasma apoB protein levels, or total cholesterol. Finally, the silencing of apoB mRNA by chol-apoB-siRNAs in liver as measured by bDNA assay and normalization to GAPDH mRNA was also demonstrated with normalization to three other liver mRNAs, including factor VII, glucose-6-phosphatase and VEGF (Supplementary Fig. 1).

Determination of *in vivo* mechanism of action

To prove that the *in vivo* activity was due to siRNA-directed cleavage, we characterized specific mRNA cleavage products using a modified 5′-RACE (rapid amplification of cDNA ends) technique previously used to demonstrate microRNA (miRNA)-directed mRNA cleavage in plants[17] and mouse embryos[18]. As it relates to the specific cleavage of apoB mRNA by apoB-1-siRNAs, total RNA from mice in the different treatment groups was isolated, and then PCR was used to reveal fragments of the predicted length in animals receiving chol-apoB-1-siRNA treatment (Fig. 6a). Identity of the PCR products was confirmed by direct sequencing of the excised bands, which demonstrated that cleavage occurred at the predicted position for the siRNA duplex. Indeed, sequencing revealed cleavage after position 10,061 of the apoB ORF, exactly ten nucleotides downstream of the 5′ end of the siRNA antisense strand. Specific cleavage fragments were detected in both liver and jejunum of animals receiving chol-apoB-1-siRNA treatment (Fig. 6b). No fragments were detected in tissues of animals receiving control siRNAs (chol-luc-siRNA or chol-mismatch-siRNA) or saline. As expected, in this 5′-RACE experiment of apoB mRNA cleavage mediated by chol-apoB-1-siRNA, no fragments were detected in tissues from animals receiving the alternative apoB-specific siRNA (chol-apoB-2-siRNA). Notably, a low level of specific cleavage product was detected in the jejunum of animals receiving the unconjugated apoB-1-siRNA despite no evidence for significant knockdown of total apoB mRNA levels by this siRNA. This indicates

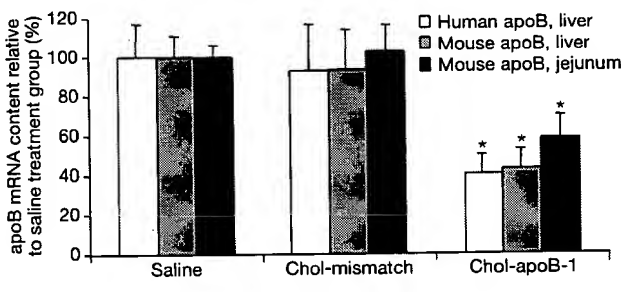

Figure 5 *In vivo* silencing of murine and human apoB mRNA in mice transgenic for human apoB. Reduction of human and mouse apoB mRNA levels in mice transgenic for human apoB that received saline ($n = 8$), chol-mismatch-siRNA ($n = 8$) and chol-apoB-1-siRNA ($n = 8$). Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline and chol-mismatch-siRNA control animals. Error bars illustrate s.d. of the mean.

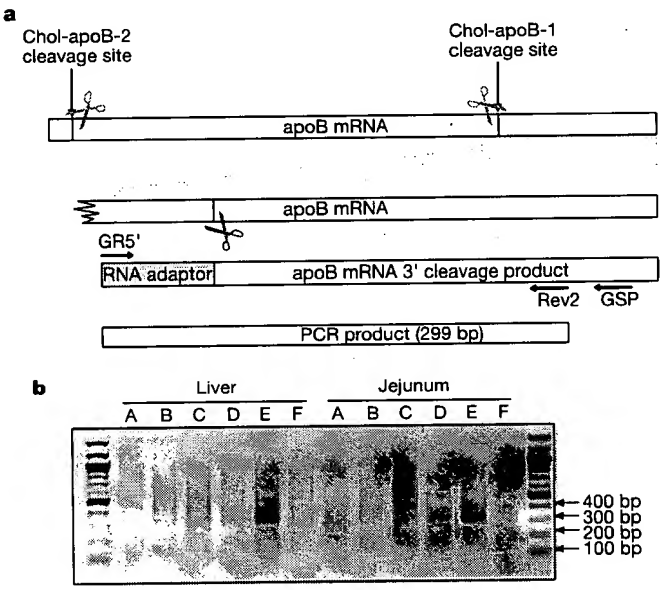

Figure 6 siRNA-mediated cleavage of apoB mRNA *in vivo*. **a**, Schematic representation of the apoB mRNA illustrating siRNA cleavage sites and RACE strategy to detect cleavage product. Cleaved mRNA ligated to an RNA adaptor was reverse transcribed using primer GSP. **b**, Agarose gel of 5′-RACE–PCR amplification, using the primer pair GR5′ and Rev2, showing specific cleavage products in liver and jejunum. Treatment groups are: A, saline; B, chol-luc-siRNA; C, chol-mismatch-siRNA; D, apoB-1-siRNA; E, chol-apoB-1-siRNA; F, chol-apoB-2-siRNA.

that some unconjugated apoB-1-siRNA is able to enter epithelial cells of the jejunum after systemic administration despite lacking cholesterol conjugation. Together, these data demonstrate that inhibition of apoB was achieved by an RNAi mechanism of action. To our knowledge, this is the first demonstration of silencing of an endogenous gene in mammals by a mechanism of RNAi-mediated degradation of the target mRNA.

Discussion

Our findings demonstrate that RNAi can be used to silence endogenous genes involved in the cause or pathway of human disease with a clinically acceptable formulation and route of administration by means of systemic delivery. In our study, we have shown that the mechanism of action for chemically modified siRNAs was by RNAi-mediated degradation of the target mRNA. Chol-apoB-siRNAs, but not unconjugated apoB-siRNAs, showed biological activity, demonstrating an important role for cholesterol conjugation of siRNAs to achieve systemic *in vivo* activity, and suggesting the opportunity to further optimize systemic activity through chemical conjugation strategies. Indeed, further optimization is warranted to achieve improved *in vivo* potency for chol-siRNAs at doses and dose regimens that are clinically acceptable. Nevertheless, these findings hold promise for the development of a new class of therapeutics that harnesses the RNAi mechanism. Of particular interest is the use of RNAi therapeutics to silence genes (such as the apoB gene) or mutated or variant alleles whose proteins are refractory to the discovery of traditional small molecules or biotherapeutic drugs. □

Methods

Synthesis of siRNAs

The siRNAs used in this study consisted of a 21-nucleotide sense strand and a 23-nucleotide antisense strand resulting in a two-nucleotide overhang at the 3′ end of the antisense strand. apoB-1-siRNA (ORF position 10049–10071): sense 5′-GUCAUCACACUGAAUACCAA*U-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAc*a*C-3′; chol-apoB-1-siRNA: sense 5′-GUCAUCACACUGAAUACCAAU*chol-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAc*a*C-3′; chol-mismatch-siRNA: sense 5′-GUGAUCAGACUCAAUACGAAU*chol-3′, antisense 5′-AUUCGUAUUGAGUCUGAUCAc*a*C-3′; chol-apoB-2-siRNA (ORF position 327–349): sense 5′-AGGUGUAUGGCUUCAACCCUG*chol-3′, antisense 5′-CAGGGUUGAAGCCAUACACCu*c*U-3′; chol-luc-siRNA: sense 5′-GAACUGUGUGUGAGAGGUCCU*chol-3′, antisense 5′-AGGACCUCUCACACACAGUUc*g*C-3′. The lower-case letters represent 2′-*O*-methyl-modified nucleotides; asterisks represent phosphorothioate linkages.

RNA oligonucleotides were synthesized using commercially available 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite monomers of uridine (U), 4-*N*-benzoylcytidine (C^{Bz}), 6-*N*-benzoyladenosine (A^{Bz}) and 2-*N*-isobutyrylguanosine (G^{iBu}) with 2′-*O*-*t*-butyldimethylsilyl protected phosphoramidites and the corresponding 2′-*O*-methyl phosphoramidites according to standard solid phase oligonucleotide synthesis protocols[19]. After cleavage and de-protection, RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography and characterized by ES mass spectrometry and capillary gel electrophoresis. RNA with phosphorothioate backbone at a given position was achieved by oxidation of phosphite with Beaucage reagent[20] during oligonucleotide synthesis. Chol-siRNAs were synthesized using the same protocols as above except that the RNA synthesis started from a controlled-pore glass solid support carrying a cholesterol-aminocaproic acid-pyrrolidine linker (V.Ke., K.G.R. and M.M., unpublished data). For this support, the first nucleotide linkage was achieved using a phosphorothioate linkage to provide additional 3′-exonuclease stability. To generate siRNAs from RNA single strands, equimolar amounts of complementary sense and antisense strands were mixed and annealed, and siRNAs were further characterized by native gel electrophoresis.

In vitro activity and stability assays

To determine *in vitro* activity of siRNAs, HepG2 cells were transfected with siRNAs using oligofectamine (Invitrogen) and siRNA concentrations ranging from 0.1, 0.3, 1, 3, 10 to 30 nM. apoB protein concentration was determined from cell culture supernatant by a sandwich ELISA capturing apoB with a polyclonal goat anti-human apoB antibody (Chemicon International). apoB detection was performed with a horseradish peroxidase-conjugated goat anti-human apoB-100 polyclonal antibody (Academy Bio-Medical Company). The remaining apoB protein content was calculated as the ratio of apoB protein concentration in the supernatant of cells treated with the apoB-specific siRNA duplex to the apoB concentration in the supernatant of cells treated with an unspecific control siRNA duplex. Mouse serum (Sigma-Aldrich Chemie GmbH) was used for stability assays. Double-stranded RNAs (5 μM) were incubated in 95% serum, and the mixture was incubated at 37 °C for various lengths of time (for example, 0, 15 or 30 min, or 1, 2, 4, 8, 16 or 24 h). siRNAs were isolated by hot phenol extraction in the presence of sodium dodecyl sulphate followed by ethanol precipitation. Re-suspended RNA samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide for 2 h at 45 mA. RNA bands were visualized by staining with the 'Stains-All' reagent (Sigma-Aldrich Chemie GmbH) according to the manufacturer's instructions.

In vivo silencing experiments

C57BL/6 mice received, on three consecutive days, tail vein injections of saline or different siRNAs. All siRNAs were administered at doses of 50 mg kg^{-1} in approximately 0.2 ml per injection. Measurements of apoB mRNA, apoB protein levels, lipoprotein concentrations and plasma cholesterol content were performed 24 h after the last i.v. injection. Experiments were carried out in a blinded fashion. The same experimental design was used for experiments with the human apoB transgenic mice (1004-T hemizygotes, Taconic).

In vivo bioanalytical methods

An RPA, using radiolabelled probes complementary to the antisense strands, was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals treated with saline or siRNAs. RPA for endogenous miRNAs was used as a loading control for jejunum (miR-143, sequence 5′-UGAGAUGAAGCACUGUAGCUCA-3′) and liver (miR-122, 5′-UGGAGUGUGACAAUGGUGUUUG-3′).

The QuantiGene assay (Genospectra) was used to quantify the reduction of mouse apoB mRNA in liver and jejunum tissue after siRNA treatment. Small uniform tissue samples where collected 24 h after the last injection. Lysates from three tissue samples per animal were directly used for apoB and GAPDH mRNA quantification, and the ratio of apoB and GAPDH mRNA was calculated and expressed as a group average relative to the saline control group. Specific probes for detection of apoB mRNA levels were designed to the following regions of the apoB mRNA ORF: probe set I 83–385; probe set II 5,045–5,673; probe set III 12,004–12,411. Furthermore, apoB mRNA reduction in liver was quantified from purified (RNeasy mRNA isolation kit, Qiagen), pooled mRNA for each treatment group. As well as GAPDH, factor VII, glucose-6-phosphatase and VEGF mRNAs were also used for normalization.

ELISA was used to quantify the reduction of apoB-100 protein levels in mouse plasma after siRNA treatment. apoB-100 from plasma samples of individual animals was detected using the primary antibody LF3 against mouse apoB-100 (gift of S. Young; see ref. [21]). Levels were normalized to plasma volume and expressed as group averages relative to the saline control group.

Total cholesterol levels in the plasma were measured using the Cholesterol detection kit (Diasys). For NMR determination of the plasma lipoprotein profile a Bruker DRX 600 with cryoprobe head was used (LipoFIT Analytic GmbH). Single measurements of 500 μl mouse plasma (pooled from ten animals per treatment group) were performed. The lipoprotein subclass distribution was calculated from the NMR data by using computer algorithms that are based on human blood standards[22]. The particle number for lipoprotein classes was calculated based on the correlation of known particle size and composition with the experimentally determined NMR signal intensity. On the basis of this correlation, the cholesterol content in the LDL fraction was computed. The cholesterol values calculated from NMR data were confirmed by the presence of comparable levels of total cholesterol in plasma and HDL-cholesterol as determined by enzymatic assays.

5′-RACE analysis

Total RNA (5 μg) from pooled liver and jejunum samples from animals treated with different siRNAs was ligated to a GeneRacer adaptor (Invitrogen) without prior treatment. Ligated RNA was reverse transcribed using a gene-specific primer (GSP: 5′-CTCCTGTTGCAGTAGAGTGCAGCT-3′). To detect cleavage products, PCR was performed using primers complementary to the RNA adaptor (GR5′: 5′-CTCTAGAGCGACTGGAGCACGAGGACACTA-3′) and apoB mRNA (Rev2: 5′-ACGCGTCGACGTGGGAGCATGGAGGTTGGCAGTTGTTC-3′). Amplification products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of specific PCR products was confirmed by sequencing of the excised bands.

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LETTERS

RNAi-mediated gene silencing in non-human primates

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The opportunity to harness the RNA interference (RNAi) pathway to silence disease-causing genes holds great promise for the development of therapeutics directed against targets that are otherwise not addressable with current medicines[1,2]. Although there are numerous examples of *in vivo* silencing of target genes after local delivery of small interfering RNAs (siRNAs)[3–5], there remain only a few reports of RNAi-mediated silencing in response to systemic delivery of siRNA[6–8], and there are no reports of systemic efficacy in non-rodent species. Here we show that siRNAs, when delivered systemically in a liposomal formulation, can silence the disease target apolipoprotein B (ApoB) in non-human primates. *APOB*-specific siRNAs were encapsulated in stable nucleic acid lipid particles (SNALP) and administered by intravenous injection to cynomolgus monkeys at doses of 1 or 2.5 mg kg^{-1}. A single siRNA injection resulted in dose-dependent silencing of *APOB* messenger RNA expression in the liver 48 h after administration, with maximal silencing of >90%. This silencing effect occurred as a result of *APOB* mRNA cleavage at precisely the site predicted for the RNAi mechanism. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and lasted for 11 days at the highest siRNA dose, thus demonstrating an immediate, potent and lasting biological effect of siRNA treatment. Our findings show clinically relevant RNAi-mediated gene silencing in non-human primates, supporting RNAi therapeutics as a potential new class of drugs.

ApoB is expressed predominantly in the liver and jejunum, and is an essential protein for the assembly and secretion of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), which are required for the transport and metabolism of cholesterol[9]. As a large, lipid-associated protein, ApoB is not accessible to targeting with conventional therapies, but it is a highly relevant and validated disease target. Elevated ApoB and LDL levels are correlated with increased risk of coronary artery disease, and inadequate control of LDL–cholesterol after acute coronary syndromes results in increased risk of recurrent cardiac events or death[10,11]. Approaches targeting ApoB with second-generation antisense oligonucleotides have progressed to pre-clinical and clinical studies[12]. Despite progress in the management of hypercholesterolaemia using HMG-CoA reductase inhibitors and other drugs that affect dietary cholesterol, there remains a significant need for new therapeutic approaches.

We have previously demonstrated silencing of *Apob* in rodents using cholesterol-conjugated siRNAs[6]. In the current study, we used a liposomal formulation of SNALP to evaluate systemic delivery of siRNA directed towards *APOB*. Preliminary evaluations were conducted in mice. Whereas administration of the *Apob*-specific siRNA siApoB-1, without formulation or chemical conjugation, at doses higher than 50 mg kg^{-1} was previously shown to have no

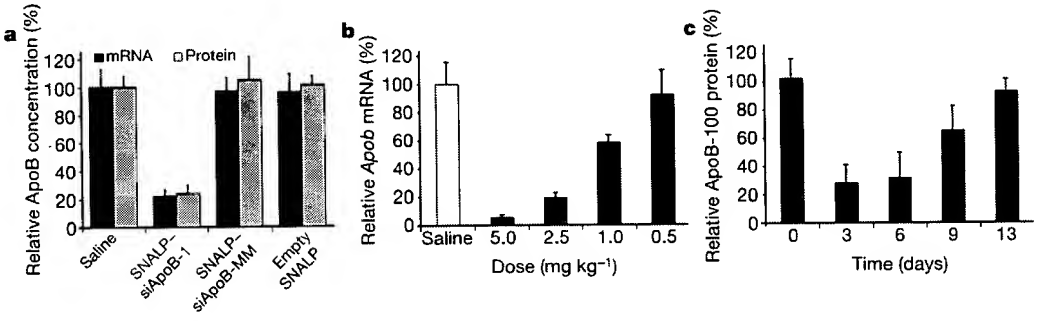

Figure 1 | SNALP–siRNA-mediated silencing of murine *Apob* is potent, specific, dose-dependent and long-lasting. a, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA and serum ApoB-100 protein levels measured two days after single i.v. injections of saline, SNALP–siApoB-1 (1 mg kg^{-1}), mismatched SNALP–siApoB-MM (1 mg kg^{-1}) or empty SNALP vesicles (25 mg kg^{-1}) ($n = 5$ per group). **b**, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA, assessed three days after i.v. administration of saline or 5, 2.5, 1 or 0.5 mg kg^{-1} SNALP–siApoB-2 ($n = 4$ per group). **c**, Serum ApoB-100 levels after i.v. administration of either saline or 2.5 mg kg^{-1} SNALP–siApoB-2 ($n = 6$ per group). Serum ApoB-100 levels for SNALP–siApoB-2-treated animals are relative to the saline-treated group for the same time point. Data show mean ± s.d.

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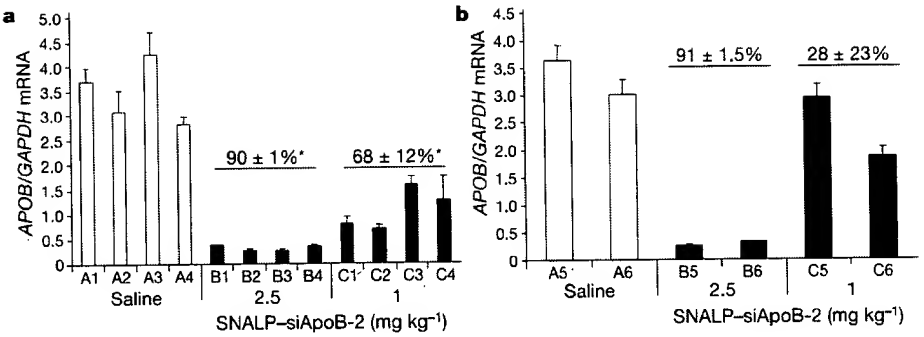

Figure 2 | Systemic silencing of *APOB* mRNA in non-human primates. **a**, **b**, Liver *APOB* mRNA levels for 12 biopsies (three isolated from each of four liver lobes) were quantified relative to *GAPDH* mRNA either 48 h (**a**, $n = 4$ animals per group) or 11 days (**b**, $n = 2$) after treatment with SNALP–siApoB-2. Data shown are mean *APOB/GAPDH* mRNA levels ± s.d. for each animal. Mean values (±s.d.) of the per cent *APOB* mRNA reduction relative to the saline treatment group are shown above each group. Asterisks indicate statistical significance compared with the saline-treated group ($P < 0.005$; ANOVA).

in vivo silencing activity[6], ~80% silencing of liver *Apob* mRNA and ApoB-100 protein was achieved with a single 1 mg kg^{-1} dose of SNALP-formulated siApoB-1 (Fig. 1a). In contrast, no detectable reduction was observed with a SNALP-formulated mismatched siRNA (siApoB-MM) or empty SNALP vesicles, indicating that silencing is specific to the siRNA and is not caused by the liposomal carrier. This silencing effect of SNALP-formulated siRNA represents more than a 100-fold improvement in potency compared with systemic administration of cholesterol-conjugated siApoB-1 (chol–siApoB-1) (Supplementary Fig. 1). Moreover, liposomal formulation of siRNA seems to be a general strategy for silencing hepatocyte targets, as demonstrated in mice for coagulation factor VII, green fluorescent protein and cyclophilin B (A.A., R. Constien and M.N.F., unpublished results).

As siApoB-1 was originally designed to be cross-reactive to both mouse and human ApoB genes, and we planned to conduct RNAi studies in non-human primates, a second ApoB-specific siRNA, siApoB-2, was designed to be cross-reactive with mouse, human and cynomolgus monkey ApoB genes. siApoB-2 was also selected on the basis of *in vitro* gene silencing activity and the absence of immunostimulatory activity (data not shown). Murine studies showed that encapsulated siApoB-2 showed a dose-dependent reduction in *Apob* mRNA, with >90% silencing achieved at the highest (5 mg kg^{-1}) dose (Fig. 1b). After a single 2.5 mg kg^{-1} dose of SNALP–siApoB-2, 80% silencing of liver *Apob* mRNA was associated with a 72% reduction in serum ApoB-100 protein. The silencing effect was detected for up to nine days, and was followed by recovery to normal protein levels by day 13 after treatment (Fig. 1c).

To address the therapeutic potential of this systemic RNAi approach, we evaluated the pharmacokinetics, efficacy and safety of SNALP-formulated siApoB-2 in cynomolgus monkeys. We first determined the circulating half-life of SNALP–siApoB-2 in plasma samples collected from cynomolgus monkeys ($n = 2$) receiving a single 2.5 mg kg^{-1} intravenous (i.v.) injection of the siRNA. An elimination half-life of 72 min was measured for the siRNA (Supplementary Fig. 2), compared with a 38-min half-life in mice (Supplementary Fig. 3a).

To evaluate efficacy, cynomolgus monkeys were treated with saline or SNALP-formulated siApoB-2 at doses of 1 or 2.5 mg kg^{-1} ($n = 6$ per group). siApoB-2 treatment was associated with a clear and statistically significant dose-dependent gene-silencing effect on cynomolgus liver *APOB* mRNA. Forty-eight hours after treatment, *APOB* mRNA was reduced by 68 ± 12% (mean ± s.d., $n = 4$, $P = 0.004$) and 90 ± 1% ($n = 4$, $P = 0.002$) for the 1 mg kg^{-1} and 2.5 mg kg^{-1} groups, respectively (Fig. 2a). Gene silencing was found to be consistent across the liver and correlated with detectable tissue levels of siApoB-2 (Supplementary Fig. 4). We also confirmed this *APOB* mRNA silencing to be mediated by RNAi, as demonstrated by 5′ rapid amplification of cDNA ends (RACE) analysis and identification of the predicted cleavage site, exactly ten nucleotides from the 5′ end of the antisense strand of siApoB-2 (Supplementary Fig. 5). Notably, *APOB* mRNA silencing was maintained for 11 days after the single 2.5 mg kg^{-1} treatment, with *APOB* mRNA levels still reduced by 91 ± 1.5% (Fig. 2b). Monkeys treated with the 1 mg kg^{-1} dose showed varying degrees of recovery from ApoB silencing at the day 11 time point. Although *APOB* mRNA was efficiently silenced in the liver, SNALP–siApoB-2 showed no silencing of *APOB* expressed in the jejunum (Supplementary Fig. 6), consistent with the absence of significant biodistribution of SNALP-formulated siRNAs to intestinal tissues in mice (Supplementary Fig. 3b).

The degree and persistence of RNAi-mediated silencing observed in cynomolgus monkeys far exceeds the results obtained with rodents. The lasting RNAi-mediated effects *in vivo* are consistent with observed long-lasting silencing by siRNAs in other studies[13,14], and the longer duration observed in primates may relate to species differences in the efficiency and stability of the RNA-induced

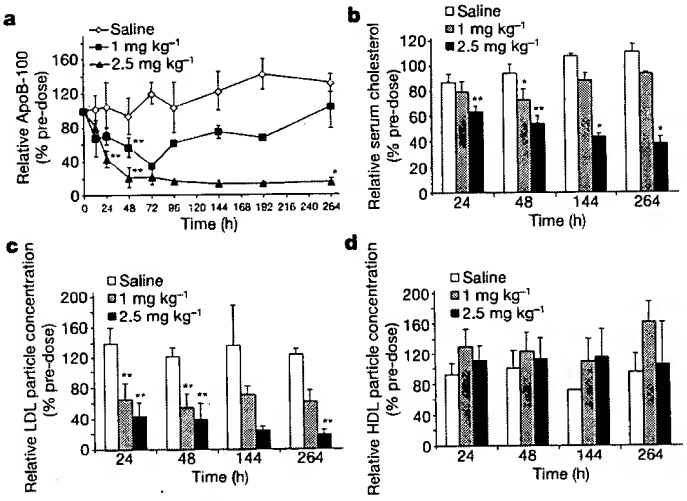

Figure 3 | Phenotypic effects of RNAi-mediated silencing of *APOB* mRNA in non-human primates. **a**–**d**, Serial plasma samples were obtained from cynomolgus monkeys treated with saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, and measured for ApoB-100 (**a**), total serum cholesterol (**b**), LDL (**c**) and HDL (**d**) levels. Data show levels as a percentage of pre-dose values and are expressed as mean ± s.d. Data sets collected at 0, 12, 24 and 48 h have a group size of six, and data sets collected at later time points have a group size of two. Data points marked with asterisks are statistically significant compared with saline-treated animals ($^*P < 0.05$, $^{**}P < 0.005$; ANOVA).

silencing complex (RISC), the mitotic state of hepatocytes and/or the tissue stability of the siRNA.

The expected biological effects resulting from *APOB* mRNA silencing include reduction in the blood levels of ApoB-100 protein, total cholesterol and LDL. To evaluate the kinetics of these downstream effects, we analysed plasma sampled serially from individual monkeys before and during the 11-day time course of the single-dose siApoB-2 study. Plasma ApoB-100 protein levels were reduced as early as 12 h after administration of 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, reaching nadirs of 35 ± 2% and 22 ± 9% of pre-treatment levels, respectively, 72 h after treatment (Fig. 3a). Animals that received the higher siRNA dose maintained a marked reduction in ApoB protein between 2 and 11 days after treatment, consistent with the lasting effect on mRNA silencing. Monkeys that received the lower siRNA dose showed an intermediate degree of ApoB protein reduction that returned to pre-dose levels by day 11, consistent with the observed recovery in *APOB* mRNA.

Serum cholesterol levels were similarly reduced, in a dose-dependent manner and with comparable kinetics (Fig. 3b). The maximum cholesterol reduction of 62 ± 5.5% ($n = 2$, $P = 0.006$) observed for the high dose siRNA group would be considered clinically significant for patients with hypercholesterolaemia, and exceeds levels of cholesterol reduction reported clinically for currently approved cholesterol-lowering drugs.

Administration of SNALP–siApoB-2 also resulted in dramatic and rapid dose-dependent reduction in the ApoB-containing lipoprotein particle LDL. Reduction in LDL relative to pre-dose levels was observed as early as 24 h after treatment for both doses of SNALP–siApoB-2 (Fig. 3c). In contrast, there were no significant changes in circulating levels of the non-ApoB-containing high-density lipoprotein particle (HDL, Fig. 3d). The reduction in LDL persisted over the 11-day study for both siApoB-2 treatment groups, with a maximum 82 ± 7% decrease compared to pre-treatment levels observed for the high-dose group at day 11 ($n = 2$, $P = 0.003$). The time required for the biological effects to return to pre-dose levels was not determined for the high-dose group because the endpoint for this study was defined using rodent data, which indicated a faster rate of recovery. The rapid onset and lasting effect on lipoprotein metabolism suggest that siRNAs targeting *APOB* may be a valuable therapeutic strategy for achieving plaque stabilization in acute coronary syndromes[10,11], as HMG-CoA reductase inhibitors can require up to 4–6 weeks to have the desired clinical effects[15].

An important consideration for the therapeutic application of siRNA relates to its general safety, as well as to the safety profile associated with specific delivery technologies. General tolerability as well as specific toxicities (such as activation of complement, coagulation and cytokines) were evaluated for all monkeys in this study. We observed no treatment-related effects on the appearance or behaviour of animals treated with SNALP–siApoB-2 compared with saline-treated animals. There was no evidence for complement activation, delayed coagulation, pro-inflammatory cytokine production (Supplementary Table 1) or changes in haematology parameters (data not shown), toxicities that have been observed previously with treatments using related approaches[16–19]. Across a systematic evaluation, the only detected change in primates treated with SNALP–siApoB-2 was a transient increase in liver enzymes in monkeys that received the high dose of SNALP–siApoB-2. The observed transaminosis peaked 48 h after treatment and was highly variable across individual animals. These effects, which were observed only at the highest dose of SNALP–siApoB-2, were completely reversible, with normalization by day 6 notwithstanding continued biological efficacy.

Our study highlights the potential for therapeutic gene silencing using systemic RNAi in non-human primates. A single, low dose of *APOB*-specific siRNA resulted in rapid and lasting RNAi-mediated gene silencing, with associated and profound phenotypic changes. The study was limited by the premature termination of the protocol after 11 days, which prevented full evaluation of the time course for RNAi-mediated effects. Although further optimization of treatment regimen and safety profile characterization may be required, our data suggest that systemic delivery of siRNAs for targeting hepatocyte-specific genes in a higher species is possible. Furthermore, the rapid and long-lasting silencing of *APOB* using RNAi may represent a new strategy for reducing LDL–cholesterol in several relevant clinical settings.

METHODS

Additional details of the methods used are provided in the Supplementary Information.

siRNA formulation. The SNALP formulation contained the lipids 3-*N*-[(ω-methoxypoly(ethylene glycol)$_{2000}$)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio.

***In vivo* experiments.** Saline and siRNA preparations were administered by tail vein injection under normal pressure and low volume (0.01 ml g^{-1}) for all rodent experiments. Cynomolgus monkeys ($n = 6$ per group) received either 2 ml kg^{-1} phosphate buffered saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2 at a dose volume of 1.25 ml kg^{-1} as bolus i.v. injections via the saphenous vein. For mRNA measurements, three liver biopsies per lobe were collected 48 h ($n = 4$) or 264 h ($n = 2$) after siRNA administration.

Bioanalytical methods. The QuantiGene assay (Genospectra) was used to quantify reduction in *APOB* mRNA levels relative to the housekeeping gene *GAPDH* in lysates prepared from mouse liver or cynomolgus monkey liver and jejunum as previously described[6] but with minor variations. Mouse[6] and cynomolgus monkey ApoB-100 protein levels were quantified by enzyme-linked immunosorbent assay (ELISA). LDL and HDL lipoprotein content were determined for plasma samples (250 µl) as described previously[6].

Statistical analysis. *P*-values were calculated for comparison of SNALP–siApoB-2-treated animals with saline-treated animals using analysis of variance (ANOVA, two-factor without replication) with an alpha value of 0.05. *P*-values less than 0.05 were considered significant.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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INTRAVITREAL INJECTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR SMALL INTERFERING RNA INHIBITS GROWTH AND LEAKAGE IN A NONHUMAN PRIMATE, LASER-INDUCED MODEL OF CHOROIDAL NEOVASCULARIZATION

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Purpose: To determine the safety and efficacy of small interfering RNA (siRNA) directed against vascular endothelial growth factor (VEGF) in a nonhuman primate model of laser-induced choroidal neovascularization (CNV).

Methods: Each animal received laser rupture of Bruch's membrane to induce CNV in both eyes. Each animal was then randomized to receive 0.05 mL of either vehicle alone or VEGF siRNA at 70 μg, 150 μg, or 350 μg in both eyes by intravitreal injection. Eyes were monitored weekly by ophthalmic examination, color photography, and fluorescein angiography for 36 days after laser injury. Electroretinograms were measured at baseline and at 5 weeks after laser. CNV on fluorescein angiograms were measured for area and graded for clinically significant leakage in a standardized, randomized, and double-masked fashion on days 15, 22, 29, and 36 after laser.

Results: VEGF siRNA did not cause any change in electroretinographic, hemorrhage, inflammation, or clinical signs of toxicity. A single administration of VEGF siRNA significantly inhibited growth of CNV and attenuated angiographic leakage in a dose-dependent manner.

Conclusion: Intravitreal injection of VEGF siRNA is capable of inhibiting the growth and vascular permeability of laser-induced CNV in a nonhuman primate in a dose-dependent manner. This study demonstrates preclinical proof of a principle that supports proceeding to clinical studies of VEGF siRNA in patients with exudative age-related macular degeneration.

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Age-related macular degeneration (AMD) is the leading cause of visual loss in the developed world and is characterized by the development of choroidal neovascularization (CNV).[1] Approved treatments such as macular photocoagulation and photodynamic therapy are limited to a subpopulation of patients and have limited efficacy.[2,3] Advances in the understanding of the pathophysiology of AMD have led to the study of novel pharmacologic treatments. In particular, several of these treatments have targeted vascular endothelial growth factor (VEGF) by binding and inactivating this protein.[4–6] However, the enclosed nature of the eye and the need to deliver a molar excess to block the effects of VEGF protein limit the dosing of these molecules based on toxicity and deliverability rather than maximum efficacy.[5,6]

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Recently, a novel method of post-transcriptional silencing of gene expression, called RNA interference (RNAi), was discovered.[7] RNAi is a conserved cellular mechanism that silences the expression of a protein in a specific and potent fashion by utilizing double-stranded RNA (dsRNA) molecules that target a particular messenger RNA (mRNA). Unlike single-stranded antisense RNA, which is designed to directly bind and inactivate a single corresponding mRNA, dsRNA binds a complex of proteins that uses the dsRNA sequence to seek and destroy homologous mRNA in a multiple turnover kinetic fashion. By activating the RNAi mechanism, one dsRNA can destroy hundreds of targeted mRNA, which will result in the silencing of potentially thousands of protein molecules.[8] Because of the potential of one molecule to suppress the expression of hundreds to thousands of protein molecules, RNAi is an ideal therapeutic strategy for the eye, which, because of its limited volume, requires potent molecules for effective local administration. Furthermore, because mRNA stabilization rather than transcription accounts for a significant proportion of VEGF upregulation,[9] post-transcriptional gene silencing by RNAi would result in a prolonged downregulation of VEGF production.

Although differing lengths of dsRNA can mediate RNAi, long dsRNA is processed by the cell to produce small interfering RNA (siRNA), a 21-nucleotide length dsRNA that acts as a true intermediate for the process of RNAi.[10] We have shown previously that siRNA designed against VEGF mRNA can silence gene expression and inhibit the development of laser-induced CNV in the mouse eye.[11] In this study, we developed a single, intravitreally delivered siRNA (Cand5) that potently targets human VEGF and has been confirmed both by sequence homology and in cell-based assays to target monkey VEGF. We used this siRNA to preclinically test its ability to inhibit laser-induced CNV in the nonhuman primate.

The laser-induced CNV model in a nonhuman primate was used to test both currently approved and investigational treatments for AMD.[12,13] The purpose of this study is to determine the toxicity of an intravitreal injection of siRNA against VEGF and to evaluate its ability to attenuate growth and leakage of laser-induced CNV in the nonhuman primate.

The RNAi mediated against VEGF is a potent strategy for inhibiting growth and leakage of CNV in macular degeneration. We demonstrate that at concentrations far below the maximum deliverable dose, one administration of VEGF siRNA can significantly inhibit both growth of CNV and VEGF-induced vascular permeability in a dose-dependent manner and with prolonged duration.

Materials and Methods

Animals

Eight adult cynomolgus monkeys (*Macaca fascicularis*), obtained from Sierra Biomedical, Reno, Nevada, were used in accordance with the guidelines of the Association of Research in Vision and Ophthalmology on the use of animals in research and according to the animal care guidelines of Sierra Biomedical. Before we performed surgical procedures, examination procedures, and electroretinography, the animals were anesthetized with intramuscular ketamine HCl at a dose of 10 mg/kg, followed with intravenous administration of ketamine and diazepam at a dose of 0.5 mg/kg. Before fundus examination or photography, the animals' eyes were dilated with 1% tropicamide.

Induction of experimental choroidal neovascularization

The CNV membranes were induced in the macular region. Twelve symmetrical spots were produced using 75 μm spot size, 0.1 second duration, and power settings ranging from 450 mW to 550 mW. To control for varying choroidal pigmentation, test spots in the peripheral retina were performed to calibrate the power required to produce a break in Bruch's membrane, indicated by the formation of a vapor bubble and small hemorrhage. Once power settings had been determined, 12 spots were placed between the vascular arcades in a symmetrical fashion. Care was taken to avoid lasering the fovea. During laser application, each spot was graded for the presence of a vapor bubble and accompanying small hemorrhage, which signified a break in Bruch's membrane. Fundus photographs were taken immediately after laser was performed. Spots that did not break Bruch's membrane were recorded and excluded from analysis. In total, only two spots were excluded.

Intravitreal injection

A siRNA molecule directed against VEGF, referred to as Cand5, was provided by Acuity Pharmaceuticals (Philadelphia, Pennsylvania). Cand5 was produced as described elsewhere.[11] Immediately after laser, eight monkeys were randomized to receive 0.05 mL of either vehicle or one of three doses of Cand5 (70 μg, 180 μg, 350 μg), which was injected into the midvitreous cavity using a 1 mL tuberculin syringe and a 30-gauge needle. The eyes were entered 2 mm posterior to the limbus. Injections were monitored for evidence of reflux or inadequate injection. If injection resulted in extensive reflux of substance, this was

recorded and eyes that had failed injections were excluded from analysis. Two eyes were excluded due to inadequate injections.

Ophthalmoscopic examination

Indirect ophthalmoscopy and slit-lamp biomicroscopy were performed before injection and weekly thereafter. Each eye was scored for evidence of inflammation using a standard inflammation grading system.[14] Slit-lamp biomicroscopy was performed to evaluate cataract formation, and the posterior pole was also examined for evidence of retinal detachment.

Electroretinogram

Electroretinograms were recorded before study and 5 weeks after laser induction. Electroretinograms were obtained with an Epic 2000 (LKC technologies, Gaithersburg, Maryland). Before electroretinography, animals were dark-adapted by being placed in a dark room for 30 minutes. After dark-adaptation, the animals were sedated and their eyes dilated. A contact lens and electrodes were placed. Calibration of the Epic 2000 was performed before testing and after testing. Photopic, scotopic, and flicker measurements were obtained using standardized light flash regimens.

Fluorescein angiography and fundus photography

Color photography and fluorescein angiography were performed with a digital fundus camera connected to the Imagenet system (Topcon TRC-50EX; Topcon, Paramus, New Jersey). Standardized photographs and fluorescein angiograms were taken for each animal at baseline and on days 15, 22, 29, and 36 after laser induction. Fluorescein angiography was performed by injecting fluorescein dye (0.1 mL/kg of 10% fluorescein sodium) intravenously and taking multiple pictures 3 seconds to 300 seconds after injection of the dye.

Fluorescein angiographic spot size measurement

The area of CNV was measured from representative early frame angiograms for each eye at each timepoint. Early frame angiograms were selected to avoid leakage seen on late frames. The representative images were selected for gradeability and represented images that were in or immediately after arteriovenous phase. The images were randomized and masked so that the reviewer was double-masked as to the animal, the time the photograph was taken, and the treatment. Using NIH Image software (Scion Corp., Frederick, Maryland), area measurements were made for each spot by tracing the neovascular area and recording the area measurement. The area measurements were performed on all lesions using a fixed calibration. To normalize the data, the area of the laser spot was subtracted from the measurement to represent the growth of CNV greater than the laser spot. Each digital image was unaltered.

Fluorescein angiogram grading

Representative early and late frame angiograms were selected from each eye for each timepoint. Early frames were selected for gradeablity and timing that demonstrated arteriovenous phase or immediately after arteriovenous phase. The range of times was from 3 seconds to 45 seconds. Late-phase angiograms were selected for gradeability and timing that was greater than 280 seconds. These images were randomized and double-masked so that the reviewer was masked to the animal, the time the photograph was taken, the time frame of the angiogram, and the treatment.

Each image was analyzed by an experienced angiogram reviewer (AJB). Each angiogram was graded for the presence or absence of CNV, presence or absence of coalescence, and presence or absence of hemorrhage. Each laser spot was graded for degree of leakage on a standardized scale of 0 to 5. Grading scores were defined as follows: 0, no hyperfluorescence; 1, mild speckled hyperfluorescent staining; 2, moderate hyperfluorescent staining; 3, mild lesion leakage; 4, moderate lesion leakage; 5, extensive lesion leakage. Clinically significant fluorescein leakage was assigned a grade of 4 or higher.

Histopathology

After the fifth-week fluorescein angiogram and electroretinogram, animals were killed under deep anesthesia and the eyes were carefully enucleated and placed in 10% formalin overnight. The eyes were then grossly dissected and the nasal aspect of the globe removed. The globe was then processed and embedded in paraffin, with care taken to orient the eye so that the area of interest (macular region) could be sectioned. Serial sections were performed and stained with hematoxylin and eosin. Slides were observed and photographed under brightfield illumination microscopy (Leica, Wetzlar, Germany) Sections were correlated with fluorescein angiograms.

Statistical analysis

For the CNV area analysis, because we are interested in the growth of CNV beyond the laser spot, we normalized all the data points to the size of the original laser spot by subtracting the area of the original

laser spot (0.24) from each CNV area measurement. The normalized CNV area measurements were treated as continuous variables and analyzed by generalized equation estimation (GEE)[15] to account for the correlations from the repeated measurements from laser spots over time from the same eye. Percentage of fluorescein leakage of grade 4 or more was determined as evidence of leakage, and the differences among Cand5 dose levels were also compared by performing GEE. All the data analysis was performed using SAS 8.2 (SAS, Cary, North Carolina).

Results

Inflammation and electroretinography

Throughout the experiment, no inflammation, cataract formation, retinal detachment, or vitreous hemorrhage was noted in any of the animals. Electroretinograms obtained at baseline and during the fifth week after laser showed no significant change between the baseline and the post-injection timepoint in any of the treated or control animals.

Area measurements

To determine the overall effect of one injection of siRNA on inhibition of laser-induced CNV, the areas of CNV for all timepoints were combined and averaged. This analysis demonstrated that all three doses of Cand5 inhibited growth of neovascular area as compared with findings in control animals ($P < 0.0001$ for 70 μg, 180 μg, and 350 μg compared with control). Although the 350 μg dose of Cand5 demonstrates a larger effect on decreasing neovascular growth than the other two dose levels, the differences are not statistically significant ($P > 0.05$). Overall, the area of CNV was reduced by more than 50% of the normalized control area when Cand5 was injected after laser injury.

To assess the efficacy of Cand5 over time, we measured growth of CNV area beyond the laser spot at days 15, 22, 29, and 36 after laser injury and intravitreal injection. At all doses and at each timepoint, the area of neovascularization was significantly lower in the treated eyes than in control eyes ($P < 0.001$ for each timepoint compared with control) (Figure 1). Thus, with one injection of Cand5, neovascular growth remains inhibited throughout the 36-day follow-up period.

Vascular permeability

In addition to size, a single injection of Cand5 was capable of decreasing leakage. Both early and late

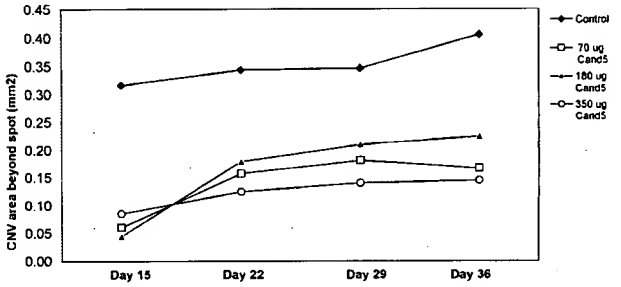

Fig. 1. Growth of choroidal neovascularization beyond the laser spot was measured from fluorescein angiograms at days 15, 22, 29, and 36 after laser induction in eyes injected with control (PBS) or one of three doses of Cand5 (siRNA targeted against VEGF). There was a significant difference between all treatment groups at each timepoint compared with control values ($P < 0.001$ for all timepoints).

fluorescein angiograms taken on day 36 demonstrate that leakage was inhibited in Cand5-treated eyes. The eye that received injection of vehicle demonstrated a large confluent bridging lesion (Figure 2A) and extensive late leakage (Figure 2B). In contrast, an eye treated with 350 μg of Cand5 demonstrated an imperceptible increase in size to the laser lesion (Figure 2C) and no late leakage of any of the laser spots (Figure 2D).

Through analysis of angiograms at each dose level, the percentage of spots that had clinically significant leakage as defined by a grade of 4 or more could be calculated for each timepoint (Figure 3). The control eyes had as much as 70% (33/47) of spots with active leakage, as compared with 27% (13/48), 6% (3/48), and 4% (1/24) of spots in eyes injected with 70 μg, 180 μg, and 350 μg, respectively, of Cand5 at Day 15; this shows a significant dose-response effect ($P < 0.0001$). Although the percentage of spots with leakage decrease with time ($P = 0.02$), the difference between treated and control eyes are still significant ($P = 0.0007$). The dose response for clinically significant leakage persists at every timepoint and can be represented as a classic pharmacologic dose-response curve (Figure 4).

To confirm that angiographically measured and graded lesions represented CNV, angiograms were correlated to histopathologic findings. Histopathology confirmed that angiographically measured CNV that stained or leaked represented subretinal neovascularization and that the extent of angiographically measured lesions corresponded to the histologic edge of the lesions (Figure 5).

Discussion

Visual loss from exudative macular degeneration results from both vascular permeability and CNV. VEGF, which is both an endothelial growth factor and

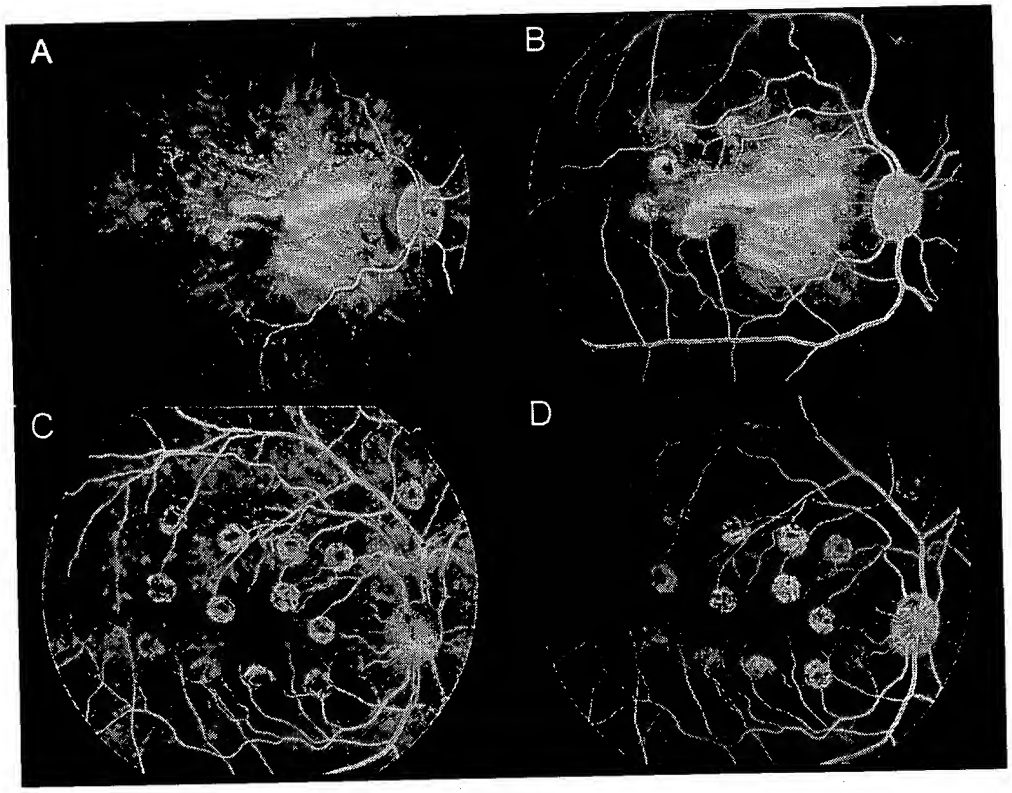

Fig. 2. Fluorescein angiograms taken 36 days after laser injury and intravitreal injection of either the control agent or Cand5. Early angiogram (during arteriovenous phase, 3 seconds) (**A**) and late angiogram (302 seconds) (**B**) of animal injected with the control agent. Early (immediately after arteriovenous phase, 30 seconds) (**C**) and late angiogram (296 seconds) (**D**) of animal injected with 350 μg of Cand5. The control animal has both large choroidal neovascularization and extensive leakage in early and late frames. The animal receiving the Cand5 shows choroidal neovascularization that does not extend past the laser spot and no leakage on late frames.

a permeability factor, plays a central role in the development of exudative AMD. By inhibiting VEGF action, both permeability and angiogenesis can be targeted. Recent clinical trials have shown that attenuating vascular permeability by the inhibition of VEGF can result in visual improvement.[6]

There are currently two inhibitors of VEGF, an aptamer and an antibody fragment, that are under commercial development for the treatment of exudative AMD. These two molecules both require direct binding of the VEGF protein on a molar-to-molar ratio to neutralize VEGF. Although both molecules are capable of inhibiting ocular neovascularization in animal models, frequent administration is required to maintain a therapeutic level of VEGF inhibition.[4,5,16] Furthermore, the requirement for frequent administration has been carried forward to the clinical trials,

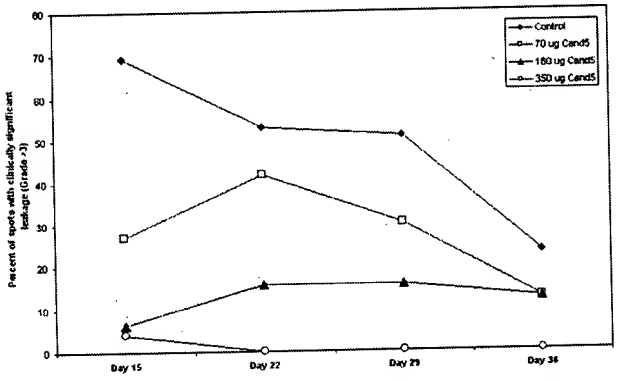

Fig. 3. Neovascular spots on angiograms were graded on a scale of 0 to 5 at days 15, 22, 29, and 36 after laser induction in eyes injected with the control agent or one of three doses of Cand5. Spots with evidence of neovascular leakage was assigned a grade of 4 or above, with the higher grade representing greater amount of leakage. At each timepoint, the percentage of neovascular spots with no significant leakage (Grade 3 or less) was higher for all three Cand5 doses as compared with control. Furthermore, increasing doses of Cand5 were associated with increasingly higher percentages of spots with no leakage.

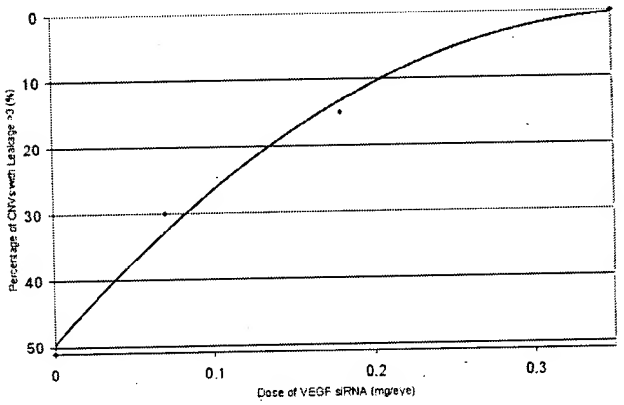

Fig. 4. The percentage of neovascular spots with no clinically significant leakage plotted against dose of Cand5 on day 29 follows a dose-response curve.

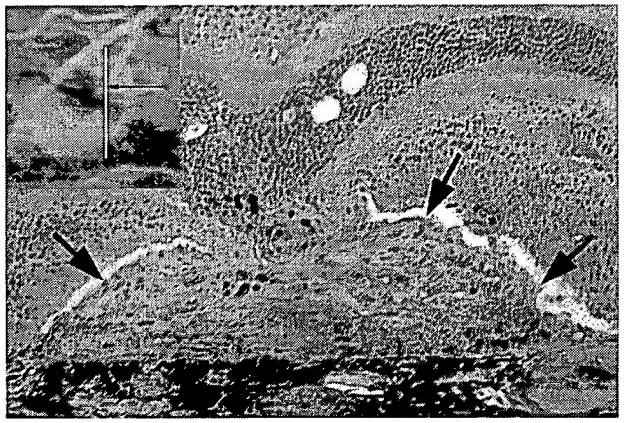

Fig. 5. Histopathologic cross section of laser-induced choroidal neovascularization that was graded with a leakage score of 3. The thin arrow and line on the angiogram shows the area ram that correlates with the tissue section. The section is stained with hematoxylin and eosin and is photographed under a ×10 microscope objective. Arrowheads point to the edge of the subretinal neovascular membrane.

which require patients to be injected every 4 to 6 weeks.

Although the advancement of these VEGF antagonists into clinical trials have validated VEGF as a therapeutic target for the treatment of AMD, the limitations of these molecules dictate the dosing and frequency of treatment. These limitations provide an opportunity to develop better VEGF inhibitors that can potentially be administered less frequently and act more potently. RNAi mediated by a siRNA against VEGF may represent this more potent and longer lasting therapeutic agent. On a molar-to-molar basis, a siRNA against VEGF is conceivably 100 to 1000 times more potent than VEGF protein antagonists. In contrast to protein antagonism, the prevention of VEGF production could potentially translate into a much longer interval between administrations, in addition to the potential for greater efficacy and decreased toxicity.

In this study, we sought to demonstrate the efficacy of RNAi in a clinically relevant model of CNV. Because of the limitations of this model and its propensity to regress spontaneously, it is not possible to directly test the full duration of effect. What we have shown in this study is that a single intravitreal injection of siRNA targeting VEGF can prevent growth of CNV and attenuate vascular leakage of CNV in a dose-dependent fashion for at least 5 weeks.

The VEGF-neutralizing fragment of monoclonal antibody against VEGF (Lucentis, Genentech, South San Francisco, California) is injected every 4 weeks and is currently under clinical investigation. This molecule was also tested in the laser-induced model of CNV in a nonhuman primate. To obtain results similar to the inhibition of leakage seen in our study, the animals required injections of rhuFAB VEGF every 2 weeks and obtained inhibition of leakage similar to our findings with a single administration of our drug.[16] With the neutralizing antibody fragment, however, the experimental eyes developed varying degrees of inflammation with their first injection and subsequent injections. In our study, we noted no inflammation, and a single injection as compared with weekly injections resulted in similar inhibition of vascular permeability, especially at our higher dose. Growth of CNV was not analyzed in the Lucentis study.

A single injection of our high dose suppressed leakage throughout the follow-up period, indicating that the duration of action on vascular permeability with one injection of Cand5 is at least 5 weeks, especially at our higher dose. Furthermore, because the maximum solubility of Cand5 in an aqueous medium is greater than 200 mg/mL (unpublished data), we are capable of dosing at much higher levels. At the dose levels used in this study, no evidence of inflammation or toxicity was noted. Formal animal toxicity studies are necessary to determine the highest acceptable dose level that can be delivered to the eye.

Although formal toxicity studies are necessary, our preliminary toxicity evaluation, which included ophthalmic examinations, histologic examinations, and electroretinograms, suggests that injections of siRNA into the eye are safe. A pegylated RNA molecule, Macugen (Eyetech Pharmaceuticals, New York, New York), which is delivered intravitreally every 6 weeks has shown efficacy in clinical trials for macular degeneration and demonstrates an excellent safety profile.[5,6] Unlike a pegylated aptamer, siRNA is a much smaller molecule, is not modified, is many-fold more soluble, and mediates a 100-fold more potent mechanism of VEGF suppression, RNA interference.

The ability of siRNA directed against VEGF to significantly inhibit choroidal neovascular growth and leakage is important for several reasons. This study illustrates the potency of siRNA as a therapeutic class of molecule. As a therapeutic agent, siRNA is potent, is highly soluble, shows no toxicity, and appears to have a longer duration of action then other available therapeutic molecules. This study also represents, to our knowledge, the first in vivo demonstrations of siRNA-mediated RNAi in a nonhuman primate administered using a clinically feasible route of administration.

In summary, this successful prevention of choroidal neovascular lesion growth and leakage in a nonhuman primate model following intravitreal administration of Cand5 is the first demonstration of therapeutic RNA interference in a primate model of disease. In addition,

this preclinical proof of principle provides a guideline for dosing in humans and provides strong support for advancing this molecule into clinical trials for the treatment of wet age-related macular degeneration.

Key words: age-related macular degeneration, choroidal neovascularization, dsRNA, nonhuman primate, RNAi, RNA interference, siRNA, small interfering RNA, vascular endothelial growth factor

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